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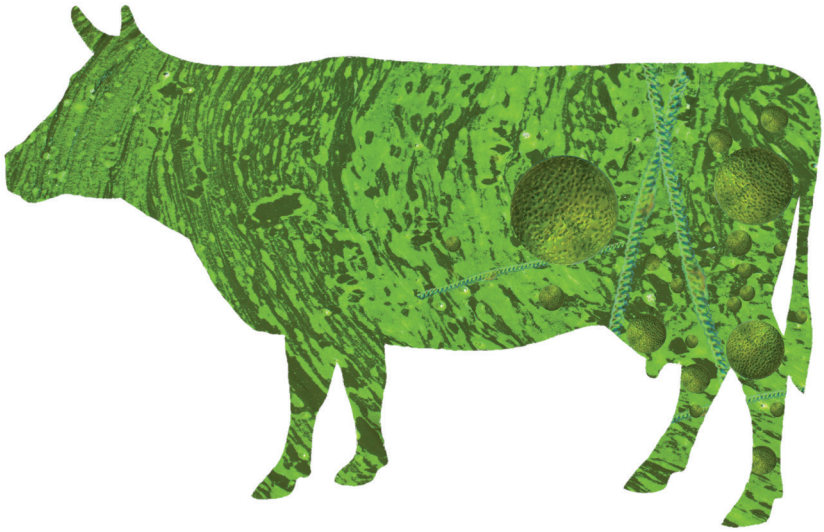
MARJUKKA LAMMINEN

POTENTIAL OF MICROALGAE TO REPLACE CONVENTIONAL PROTEIN FEEDS FOR SUSTAINABLE DAIRY COW NUTRITION



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University of Helsinki
Finland

POTENTIAL OF MICROALGAE TO REPLACE CONVENTIONAL PROTEIN FEEDS FOR SUSTAINABLE DAIRY COW NUTRITION

Marjukka Lamminen

ACADEMIC DISSERTATION

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Supervisors: **Docent Seija Jaakkola**
Department of Agricultural Sciences
University of Helsinki, Finland

Professor Aila Vanhatalo
Department of Agricultural Sciences
University of Helsinki, Finland

Docent Tuomo Kokkonen
Department of Agricultural Sciences
University of Helsinki, Finland

Dr. Anni Halmemies-Beauchet-Filleau
Department of Agricultural Sciences
University of Helsinki, Finland

Pre-examiners: **Professor Alexander Hristov**
Department of Animal Science
The Pennsylvania State University, the United States

Professor Glen Broderick
Department of Dairy Science
University of Wisconsin, the United States

Opponent: **Associate Professor Jan Dijkstra**
Department of Animal Sciences
Wageningen University and Research, the Netherlands

Custos: **Professor Aila Vanhatalo**
Department of Agricultural Sciences
University of Helsinki

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Kyllä minä muistan:
sehän oli kesäkuuta
maa kukki
olin kuusivuotias.

Isä talutti kirkonkylältä
huutokaupasta lehmän:
siinä on, opettele lypsämään.
Ja sain sangon
ja sain jakkaran,
ja kohta putoili maito pehmeinä paloina
keltainen kesämaito
apilasta koottu.

Päivällä juoksin laitumelle
että mitenkä se minun Mansikkini?

Siellä se haukkasi heinää
sen korvat keikkuivat kärpäsille
ja hännällä se tuuletti kupeitaan.

-Minun kulta Mansikkini, minä sanoin
ja puristin sitä kaulasta.
-Minun kulta Mansikkini, minä sanoin
ja suutelin sitä.

Vielä veräjällä minä käännyin katsomaan:
suurelta lihavalta kukalta se näytti.

Eeva Heilala, Punaposkipuolukoita. Valitut runot. Tammi 2004.

CONTENTS

ERRATUM

p. 7: In two out of four experiments, these changes did not convert to major changes in arterial concentrations of these AA and their metabolites. The changes observed with *S. platensis* or mixture of *S. platensis* and *C. vulgaris* were decreased (Exp. 1; I) or tendency for decreased (Exp. 2; I) arterial histidine concentration, decreased arterial carnosine concentration in Exp. 1 and 2 (I), and decreased mammary uptake of histidine in Exp. 2 (I) and methionine in Exp. 4 (III).

p. 24, 57: Correct spelling *Arthrospira platensis*.

ABSTRACT

The objective of the research described in this thesis was to evaluate the potential and sustainability of protein feed use of different non-defatted microalgae species in the nutrition of lactating dairy cows. Four physiological experiments (Publications I-III) were conducted with dairy cows to compare dry matter (DM) intake, milk production, energy metabolism, nitrogen (N) utilisation and amino acid (AA) metabolism using diets containing microalgae (*Spirulina platensis, Chlorella vulgaris* and *Nannochloropsis gaditana*) and conventional protein feeds (rapeseed meal, soya bean meal and faba beans). The microalgae diets constituted of *S. platensis* (Exp. 2, 3 and 4; I-III), *C. vulgaris* (Exp. 3; II), mixture of *S. platensis* and *C. vulgaris* (Exp. 1; I), and mixture of *C. vulgaris* and *N. gaditana* (Exp. 3; II). These microalgae substituted protein in rapeseed meal partially (Exp. 1, 2 and 4; I, III) or completely (Exp. 1 and 2; I), soybean meal completely (Exp. 3; II), and faba beans partially (Exp. 4; III). In addition, the effect of protein supplementation with rapeseed meal and microalgae in comparison to unsupplemented diet (Exp. 2; I) was studied.

The crude protein (CP; Kjeldahl-N × 6.25) concentration of microalgae was on average 690 g/kg DM for *S. platensis*, 597 g/kg DM for *C. vulgaris* and 385 g/kg DM for *N. gaditana*. These microalgae were relatively low in crude fat, with highest concentration of 192 g/kg DM measured for *N. gaditana*. Compared to conventional protein feeds, all microalgae were lower in histidine, but higher in methionine.

The DM intake (DMI) of microalgae containing feeds was lower than that of conventional protein feeds in three out of four experiments. When forage and concentrates were fed separately, cows compensated the decreased intake of microalgae containing concentrates by increasing silage intake (Exp. 2 and 3; I-II). Thus, total DMI remained unchanged (Exp. 2 and 3; I, II). This decreased the proportion of concentrate in the diet as much as 11.4 %-units. When all feed components were offered as total mixed ration (TMR), total DMI was decreased (on average 0.65 kg/d) as cows no longer were able to avoid microalgae (Exp. 4; III). The poor palatability of microalgae was not related to amount of microalgae in the diet, dietary crude fat concentration or stage of lactation.

Milk yield was unaffected by microalgae inclusion in the diet in three out of four experiments. The partial substitution of rapeseed meal or faba beans with *S. platensis* decreased milk yield on rapeseed, but increased on faba bean supplemented diets (Exp. 4; III). The same was observed as a tendency for protein yield. Also in Experiment 2 (I), *S. platensis* tended to result in lower milk protein yield than rapeseed meal. Microalgae resulted in milk, energy corrected milk (ECM) and protein yields similar to soya bean meal (Exp. 3; II). *S. platensis* inclusion in the diet increased milk fat concentration on soya (Exp.

3; II) and rapeseed meal (Exp. 4; III) supplemented diets, which might have been caused by more acetate intensive ruminal fermentation, increased body mobilisation or higher methionine intake.

Protein supplementation increased N excretion in urine and faeces, and decreased the conversion efficiency of dietary N into milk protein (milk N:N intake; NUE) (Exp. 2; I). The responses of microalgae inclusion in the diet depended on the source of protein in the basal diet. In Exp. 2 (I), the substitution of rapeseed meal with *S. platensis* decreased NUE and human-edible protein efficiency. The same was observed in Exp. 4 (III) on rapeseed meal supplemented diets, whereas on faba bean supplemented diets NUE was increased and human-edible protein efficiency was unaffected by *S. platensis* inclusion in the diet. In addition, the partial substitution of both rapeseed meal and faba beans resulted in increased urinary and total excretion of N (Exp. 4; III). The complete substitution of soya bean meal with different microalgae did not affect NUE, but decreased urinary N excretion and tended to increase human-edible protein efficiency.

The substitution of all conventional protein feeds with microalgae decreased the intake of histidine and increased that of methionine. In three out of four experiments, these changes did not convert to major changes in arterial concentrations of these AA and their metabolites. The changes observed with *S. platensis* inclusion in the diet were tendency for lower arterial histidine concentration in Exp. 2 (II), decreased mammary uptake of histidine in Exp. 2(II) and methionine in Exp. 4 (III).

In conclusion, the results demonstrated no biological or physiological constraints for protein feed use of different microalgae. Moreover, microalgae seemed to be suitable substitute for soya bean meal and faba beans in dairy cow nutrition. However, microalgae are slightly inferior to rapeseed meal as indicated by results of milk production and N utilisation. The greatest challenge limiting the feed use of microalgae for lactating dairy cows is their poorer palatability relative to conventional feeds.

Keywords: Microalgae, dairy cow, milk production, amino acid metabolism, nitrogen utilisation, *Spirulina platensis*, *Chlorella vulgaris*, *Nannochloropsis gaditana,* rapeseed meal, soya bean meal, faba beans.

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Together with this research team, we have sweated over the hands-on-work in cow barn and laboratory, and shared the joys of scientific discoveries and formulating new hypotheses, and the frustration of days when nothing works as it should be. Yet, I hope that we never again have to design a completely new experiment in one week out of thin air after geese and rats have destroyed the original experimental feed material. Oh, the joys of science!

I also wish to express my gratitude to all my old and new friends near and far, who have supported me on this project. My warm thanks go to my my mother and father who took me with their daily chores in our family farm when I was little and taught me to appreciate hard work, nature, animals, and food produced. I thank my beloved Ismo for being the most wonderful companion on this journey and all other journeys. Thank you for your love, understanding, encouragement and support, and the movie nights – I could not have done this without you! Your belief on me and trust on my choices have been a firm

foundation for finding my own path in this world. When I'm with you, I'm standing with an army.

Thank you all for making this journey possible!

Wageningen, 19 October 2018

Marjukka Lamminen

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on work reported in the following original publications subsequently referred to in the text by their Roman numerals:

I Lamminen, M., Halmemies-Beauchet-Filleau, A., Kokkonen, T., Simpura, I., Jaakkola, S. & Vanhatalo, A. 2017. Comparison of microalgae and rapeseed meal as supplementary protein in the grass silage based nutrition of dairy cows. *Animal Feed Science and Technology* 234: 295-311.

II Lamminen, M., Halmemies-Beauchet-Filleau, A., Kokkonen, T., Jaakkola, S. & Vanhatalo, A. 2019. Different microalgae species as a substitutive protein feed for soya bean meal in grass silage based dairy cow diets. *Animal Feed Science and Technology* 247: 112-126.

III Lamminen, M., Halmemies-Beauchet-Filleau, A., Kokkonen, T., Vanhatalo, A. & Jaakkola, S. The effect of partial substitution of rapeseed meal and faba beans by *Spirulina platensis* microalgae on milk production, nitrogen utilization and amino acid metabolism of lactating dairy cows. *Journal of Dairy Science* (in press).

AUTHORS' CONTRIBUTION

The contributions of all authors to the original publications of this thesis are described in Table 1 (initials of authors are listed in alphabetical order).

Table 1. The contributions of authors to the publications.

	Publications		
Phase of work	**I**	**II**	**III**
Planning the experiment	AH, AV, ML, SJ, TK	AH, AV, ML, SJ, TK	AH, AV, ML, SJ, TK
Conducting the experiment	AH, ML, TK	AH, ML, TK	AH, ML, SJ, TK
Laboratory analysis	AH, IS, ML	AH, ML	AH, ML
Data analysis	AH, ML	AH, ML	AH, ML
Drafting the first version of manuscript	ML	ML	ML
Commenting and modifying the manuscript	AH, AV, IS, ML, SJ, TK	AH, AV, ML, SJ, TK	AH, AV, ML, SJ, TK

AH = Anni Halmemies-Beauchet-Filleau
AV = Aila Vanhatalo
IS = Ilkka Simpura
ML = Marjukka Lamminen
SJ = Seija Jaakkola
TK = Tuomo Kokkonen

ABBREVIATIONS

AA	Amino acid
AA-N	Amino acid nitrogen
ADF	Acid detergent fibre
AIA	Acid-insoluble ash
BHBA	β-hydroxybutyrate
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
EAA	Essential amino acid
ECM	Energy corrected milk
EPA	Eicosapentaenoic acid
EU	European Union
FA	Fatty acid
GLA	ɣ-linolenic acid
iNDF	Indigestible neutral detergent fibre
IUPAC	International Union of Pure and Applied Chemistry
ME	Metabolisable energy
MUFA	Monounsaturated fatty acid
MUN	Milk urea nitrogen
N	Nitrogen
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acids
NPN	Non-protein nitrogen
NUE	Nitrogen use efficiency in milk production (milk N:N intake)
OM	Organic matter
PUFA	Polyunsaturated fatty acid
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SFA	Saturated fatty acid
sp.	Species (singular)
spp.	Species (plural)
TMR	Total mixed ration
VFA	Volatile fatty acid

1 INTRODUCTION

Ruminant animals fill a unique evolutionary niche with their capability of being independent of dietary supply of true protein and to convert non-protein-N (NPN) such as urea to high quality milk protein (Virtanen, 1966). While this potential is broadly been neglected in modern intensive milk production relying too much on supply of human-edible feed components in cow diets, the feeding of cows with protein supplements is reasonable on many grounds. First, the ruminal microbial production *per se* is insufficient to satisfy the protein requirements of high yielding modern dairy cows in intensive milk production (Santos et al., 1998). Second, the use of urea in ruminant diets is rather limited because of its rapid hydrolysis to ammonia, which may lead to hyperammonemia if given in excess to the capacity of ruminal microbes to synthesise AA and proteins (Cope, 2012). Moreover, ruminal microbes fermenting non-structural carbohydrates respond positively to the provision of peptides and AA (Russell et al., 1992). Thus, the supply of dietary protein and peptides can be more efficient in stimulating microbial protein production in the rumen than NPN as a sole N source in the diet. Indeed, dairy cow diets based on various true protein sources resulted in higher milk production levels and conversion of feed DM and N into milk and milk protein than diet based on urea (Brito and Broderick, 2007). Similarly, protein supplementation in general increases DMI (e.g. Oldham, 1984; Allen, 2000; Huhtanen et al., 2011), and milk yield and milk protein concentration (Huhtanen et al., 2011).

1.1 THE SUSTAINABLE PROTEIN NUTRITION OF DAIRY COWS

Feed production and animal feeding are the main processes in any animal production system that determine to large extent the emissions of N, phosphorus (P) and greenhouse gases to environment, but also affect animal health and welfare, and the hygienic quality and nutritive value of animal derived products. Therefore, the sustainable feed production and animal feeding is the prerequisite of achieving resource efficient and resilient livestock production systems that result in minimal environmental load and high product quality (Makkar, 2016).

The following three points are relevant when aiming to improve the sustainability of protein feeding practices: First, the selection of appropriate protein feed matters because these feeds vary in their environmental impact and milk production response. Second, attention should also be paid to support local production of protein feeds. The protein self-sufficiency of European Union (EU) is only around 30% when considering the feed use of

soya beans (*Glycine max*), rapeseed (*Brassica napus* subsp. *oleifera*), sunflower (*Helianthus annuus*), other oilseeds and pulses (Bouxin, 2017). This decreases the security of supply of European livestock sector and makes it vulnerable to trade distortions, availability and price volatility of imported protein feeds, especially soya bean meal (Häusling, 2011, de Visser et al., 2014). Third, the optimisation of dietary CP concentration in relation to energy supply is crucial, since intake of N is directly linked to excretion of N to environment (e.g. Fisher et al., 2000; Korhonen et al., 2002, Broderick, 2003) and NUE (e.g. Broderick, 2003; Huhtanen et al., 2008a; Spek et al., 2013). In ruminants, protein and energy metabolism are interconnected processes, and the insufficient ruminal balance of N and energy can limit the utilisation of feed nutrients. Moreover, protein feeds are typically the most expensive feed component in dairy cow diet and overfeeding of protein is not economically wise due to diminishing ratio of marginal returns, i.e. reducing milk production response to increasing protein supplementation (e.g. Broderick, 2003; Olmos Colmonero and Broderick, 2006). Thus, finding optimal dietary CP concentration can be considered to have direct benefits to environment and farm economy.

1.2 THE EVALUATION OF THE SUSTAINABILITY OF FEED PRODUCTION AND FEEDING PRACTICES

The sustainable animal diet has been determined as one that is balanced in all nutrients, free from harmful components, meets production objective, generates animal products safe for human consumption, and integrates the environmental, economic and societal aspects of sustainability (Makkar and Ankers, 2014). When evaluating the sustainability of a certain animal feeding practice, the three dimensions of sustainability (environment, economy, society) are good starting principle. However, when working with living beings, the inclusion of ethics as a fourth dimension of sustainability is also required (Rawles, 2010; Figure 1). The dimensions of environmental sustainability of milk production can be further divided into e.g. N and P load to environment, fresh water use, land-use changes induced, ecological and material footprints and greenhouse gases emitted (Figure 2). Of these seven environmental sustainability dimensions, this thesis focuses on the N utilisation of intensive milk production. The N utilisation is evaluated from three different perspectives: efficiency, sufficiency and consistency. This triple approach suggested here builds on the work of Huber (2000) originally proposing the approaches of efficiency, sufficiency and consistency for sustainable development, White et al. (2016) describing modern socio-environmental debate, and Garnett et al. (2014) outlining perspectives on sustainable food security.

The triple approach was chosen to cover different aspects of N utilisation that all contribute to the environmental performance of milk production. In

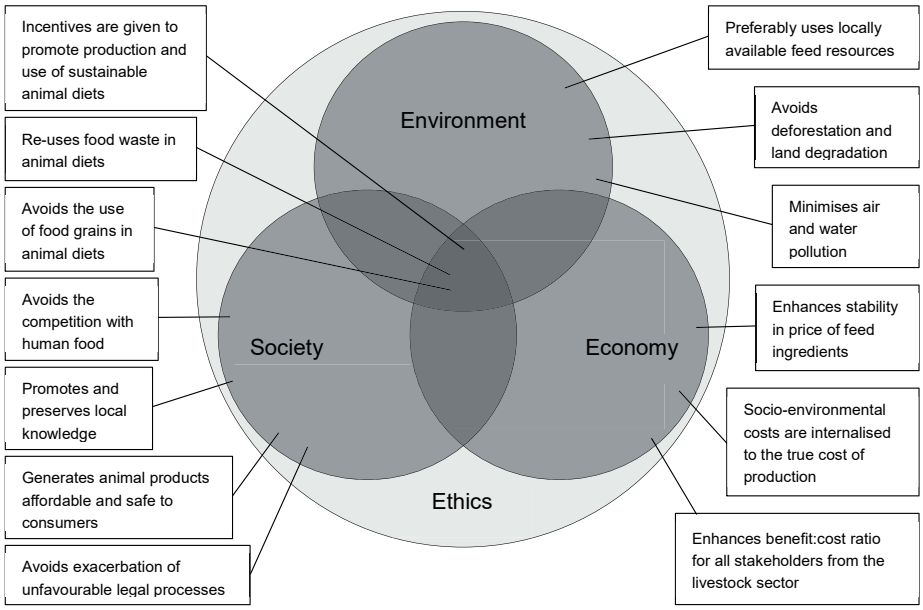
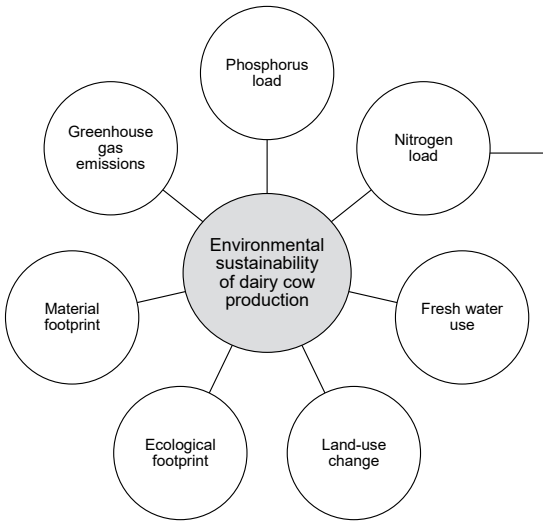


Figure 1 Some characteristics of sustainable feed production. Adapted from Makkar and Ankers, 2014.



The sustainability of nitrogen (N) utilisation in milk production		
Efficiency	Sufficiency	Consistency
N output in milk (kg) per N input in animal feed (kg)	N excretion in urine and faeces (kg) per animal	Human-edible output of protein (kg) in milk per human-edible input of protein (kg) in animal feed

Figure 2 The environmental sustainability dimensions of dairy cow production (adapted from O'Neill et al., 2018), three strategies to increase sustainability (efficiency, sufficiency and consistency; Huber, 2000), and suggestion of metrics to measure the sustainability of nitrogen utilisation in milk production.

the efficiency strategy, the focus of attention is in production processes, and the aim is to decrease the environmental load by improving the input:output ratio of certain production system via optimisation, and technological and management solutions. In the case of protein feeding of dairy cows, this would mean e.g. optimising dietary N:metabolisable energy (ME) ratio and balancing AA composition in order to maximise the conversion efficiency of feed N into milk protein. In the sufficiency strategy, the focus of attention is in consumption side, and the aim is to decrease the environmental load by decreasing consumption of certain products. In the case of protein feeding of dairy cows, this would mean e.g. minimising the amount of protein fed to cows to decrease amount of N emitted to environment. In the consistency strategy, the focus is on the structures of food system and quality of products, and the aim is to decrease the environmental load of a certain production system via better integrated and ecologically more sensible production chains. In the case of milk production, this would mean e.g. adaptation of circular economy practices in milk production chain and feeding animals with human-inedible feed components. Thus, the triple approach adapted in this thesis in regards N utilisation include the measurements of N output in milk per N input in feed (NUE), N emitted in environment via urine and faeces, and conversion of human-edible protein in feed into human-edible protein in milk (Figure 2).

Of these metrics, efficiency (i.e. output:input ratio of N) and sufficiency (i.e. quantity of N emissions to environment) are most commonly reported in dairy cow research. Indeed, at best, efficiency and sufficiency oriented strategies can decrease environmental load and increase resource use efficiency by improving the weak points of the production system. However, neither of these approaches takes into account the contribution of animal production to food security, since they lack tools to quantify the effects of nutritional quality of feed on animal products generated. In addition, risks are related relying particularly only on efficiency based metrics when evaluating the sustainability of any production system. First, the efficiency-based strategies to improve environmental sustainability of any production system are susceptible to purchase-resale bias. This bias comes from the mathematical phenomena that the output:input ratio changes when both numerator and denumerator are increased with the same amount (Godinot et al., 2014). The direction of this change (increase or decrease of output:input ratio) depends on the input and output values. When input is larger than output, like in the ratio of milk N:N intake, output:input ratio increases when both input and output is increased with the same amount. This means that a cow with 1 g/d higher production of milk N and 1 g/d higher N intake has higher NUE than a cow with 1 g/d lower milk N production and 1 g/d lower N intake. On the other hand, when output is larger than input, like in the ratio of ECM:DMI, output:input ratio decreases when both input and output is increased with the same amount. This means that a cow with 1 kg/d higher DMI and 1 kg/d higher ECM yield has lower ECM:DMI ratio than a cow with 1 kg/d lower DMI and 1 kg/d lower ECM yield. This phenomenon is likely less strong for NUE than

ECM:DMI ratio because physiologically NUE decreases with increasing N intake. Second, also the rebound effect can hinder or even completely prevent true sustainability gains of efficiency based strategies, if not taken into account. Simply put, the rebound effect refers to a situation where the potential energy or raw material savings that could be achieved with more efficient production systems are not realised because the decreased costs per unit of service or product leads easily to increased consumption (Berkhout et al., 2000). Thus, the process itself is more efficient, but since the total production is increased, the environmental load of production might not change. Moreover, in many cases the increased resource use efficiency increases the intensity of the production system, which can have multiple other harmful environmental effects that are not necessarily taken into account if the sustainability of the production system is not evaluated from the holistic perspective. Therefore, together with efficiency and sufficiency, simultaneous implementation of a third strategy, consistency (i.e. human-edible feed conversion efficiency in the case of this thesis), is the prerequisite for achieving holistic understanding of the sustainability of N utilisation.

1.3 CONVENTIONAL AND ALTERNATIVE PROTEIN FEEDS FOR SUSTAINABLE DAIRY COW NUTRITION

The efficiency of milk production and N emissions caused can be affected by choosing appropriate protein feed to balance the composition of basal diet. Protein feeds differ in e.g. AA composition and ruminal protein degradability, which can affect their milk production responses. Balancing AA supply is beneficial since it has increased milk and milk protein yields, milk protein concentration and NUE (Haque et al., 2012). The critical AA most often limiting milk production are especially methionine (e.g. Schwab et al., 1976; Pisulewski et al., 1996; Socha et al., 2005), lysine (e.g. Schwab et al., 1976; Socha et al., 2005) and histidine (e.g. Kim et al., 1999; Vanhatalo et al., 1999). The ruminal degradability of protein is a key characteristic of any feed, and it determines the availability of undegraded AA for intestinal absorption but also affects the efficiency of conversion of feed N into milk protein. If the supply of rumen degradable protein (RDP) exceeds the requirements of rumen microbes or if the dietary supply of fermentable carbohydrates is insufficient in relation to RDP, the excess N is largely converted to NH_4^+ in rumen, transported into liver for conversion to urea, and excreted in the urine and milk (Figure 3). On the other hand, suboptimal RDP supply can limit ruminal microbial protein production and carbohydrate digestibility, leading to lower milk production response. Thus, trade-offs of maximal ruminal microbial efficiency and minimal environmental N load are related to finding optimal balance between RDP and rumen undegradable protein (RUP) (Reynal and Broderick, 2005).

1.3.1 RAPESEED MEAL AND GRAIN LEGUMES

Rapeseed and grain legumes such as soya bean and faba beans (*Vicia faba*) are protein feeds conventionally used in dairy cow diets, of which rapeseed and soya beans constitute around 81% of the protein-rich feed materials consumed in EU (Bouxin, 2017). Especially rapeseed meal low in glucosinolates is generally considered high quality protein feed that is very well suited to dairy cow nutrition (Huhtanen et al., 2011; Martineau et al., 2013; Broderick et al., 2015). In addition, rapeseed has resulted in higher NUE (Shingfield et al., 2003; Broderick et al., 2015; Gidlund et al, 2015; Rinne et al., 2015) and human-edible feed conversion efficiency (Karlsson et al., 2018), and lower urinary N losses (Shingfield et al., 2003; Broderick et al., 2015; Rinne et al., 2015; Paula et al., 2018) than soya beans in dairy cow nutrition.

However, sustainability problems are related to production and utilisation of these two conventional protein feeds. Neonicotinoid insecticides are commonly used in cultivation of rapeseed to protect the plant from pests. Growing body of research has shown neonicotinoids to be harmful chemicals not only for pollinators but also many other living organisms (Wood and Goulson, 2017). Both the disruption of pollination services by neonicotinoids, and recent restrictions of the use of neonicotinoids in EU have been linked to possibly decreasing rapeseed yields (Budge et al., 2015; Hokkanen et al., 2017). Another sustainability concern related to rapeseed cultivation is the positive N balance in soil in crop rotations including rapeseed, and high N leaching to environment is often measured in the winter following rapeseed cultivation (Nielsen and Jensen, 1990; Christen, 2012). However, this problem is not unique to rapeseed cultivation, since substantial N leaching to environment is also measured after the cultivation of grain legumes without the use of catch crops (Hauggaard-Nielsen et al., 2009). Even so, Nielsen and Jensen (1990) reported lower N leaching for grain legumes than for rapeseed.

Grain legumes such as soya bean meal and faba bean are typically low in methionine and have high ruminal protein degradability (Luke, 2018), which hinders their value as protein feeds for ruminants. Both of these leguminous crops utilise biological N fixation, thus decreasing reliance on fossil-fuel intensive N fertilisation. However, in terms of soya bean meal, sustainability concerns are related to global trade leading to geographically very far diverged production and consumption of protein feeds. This makes the recycling of nutrients impossible. Thus, the massive nutrient flows lead to enrichment of nutrients on consumption site but impoverishment on the production site, which must then be compensated by fertilisation or biological N fixation (Smaling et al., 2008). Moreover, soya bean production in South America is the direct and indirect driver of the destruction of rainforest and savannah ecosystems, which causes greenhouse gas emissions, biodiversity loss, destruction of living areas of especially indigenous people, and disruption of atmospheric water flows leading to increasingly dry conditions (Smaling et al., 2008).

Faba beans are currently underutilised as protein source for livestock nutrition. However, they have huge potential especially in North European conditions, where they outyield other conventional protein feeds (Laine et al., 2017). Moreover, the cultivation of faba beans have multiple environmental benefits that go beyond biological N fixation. It contributes to regulating and supporting ecosystem services with diversifying crop rotations leading to richer biodiversity and lower pest and disease pressure, and improving soil fertility and carbon storage in soil (Watson et al., 2017). However, in dairy cow diets faba beans have induced lower milk production response than rapeseed meal (Puhakka et al., 2016) or rapeseed expeller (Ramin et al., 2017). Also the urinary N losses have been higher for faba beans than for rapeseed meal (Puhakka et al., 2016). This is notable because urinary N is the most susceptible form of N for environmental leaching (Bussink and Oenema, 1998). Supplementing faba beans with methionine-rich feeds may be beneficial. The benefits of methionine supplementation on soya bean meal supplemented dairy cow diets include increased DMI and fat corrected milk yield (Schingoethe et al., 1988; Broderick et al., 2009), and milk protein yield and concentration (Schingoethe et al., 1988; Pisulewski et al., 1996; Armentano et al., 1997; Broderick et al., 2009). Some experiments have also shown potential to decrease dietary CP concentration with methionine supplementation without adverse effects to milk production (Broderick et al., 2008). However, these beneficial effects of methionine supplementation of soya bean diets have not been found in all experiments (e.g. Casper et al., 1987; Casper and Schingoethe et al., 1988; Guillaume et al., 1991).

1.3.2 MICROALGAE

Given the environmental and compositional challenges related to production and use of conventional protein feeds, novel feed resources are warranted, the production and utilisation of which is environmentally and socially sustainable, and the feeding of which generates high-quality animal products. Microalgae are feed material that could possibly fill these criteria. Microalgae are mostly photosynthetic, unicellular or simple multicellular microorganisms that grow in widely varying environmental conditions, not only in aquatic but also terrestrial ecosystems (Mata et al., 2010). A distinct characteristic of algae in general are their huge diversity in numbers and types of species and their chemical composition. Indeed, algae species are found in four different biological kingdoms: Bacteria, Plantae, Chromista and Protozoa (Guiry, 2012). Moreover, the estimates of amount of algae species vary from 30 000 to more than a 1 million species (Guiry, 2012). Currently, there are around 153 000 species of algae (micro and macroalgae) listed in taxonomic database AlgaeBase (Guiry and Guiry, 2018). For the purposes of this thesis, term microalgae is used to refer both prokaryotic species such as cyanobacteria (*Cyanophyceae*) and eukaryotic species such as green algae (*Chlorophyta*) and *Ochrophyta* with microscopic size.

Microalgae have several advantages over terrestrial feed resources. First, they grow extremely rapidly commonly doubling their biomass within 24h or less (Chisti, 2007) leading to very short harvesting cycle of 1 to 10 days (Schenk et al., 2008). Moreover, as the microalgal CP concentration can reach up to 710 g/kg DM (Becker, 2013), very high protein yields exceeding 2 to 25 times those of conventional protein feeds (rapeseed, soya bean and other grain legumes) have been reported on area basis in North-western Europe (van Krimpen et al., 2013). In addition, marginal or non-arable land can be used in microalgae cultivation, which could allow vast areas of land to be repurposed for human consumption (Schenk et al., 2008). In theory, zero land-use change could be achievable with the cultivation of microalgae in floating photobioreactors in marine environment (Wiley et al., 2013). For the above-mentioned reasons, microalgae cultivation can be considered to have potential to improve food security (Efroymson et al., 2017). Despite of microalgal cultivation in aquatic environment, the water footprint of microalgae utilised for biofuel production is generally markedly lower than that of soya bean due to efficient water recycling in microalgae production (Batan et al., 2013). Moreover, fresh water is not necessarily needed in microalgae cultivation, instead salt water or wastewaters can be used, the practice of which can lower production costs of microalgae and provide wastewater treatment services (Usher et al., 2014). Nevertheless, the large-scale feed use of microalgae is still hindered by their high production cost (Pang et al., 2018), but the competitive position of microalgae in relation to conventional feed resources can change in the near future due to e.g. rapid technological development in microalgal production and different policy interventions such as incentives and carbon taxation.

Algae and different algae products are already included in the European Community Catalogue of Feed Materials (Commission Regulation (EU) No. 68/2013), so their usage is allowed for animal feeding purposes. However, little is known about the value of microalgae as protein feeds for ruminants. Instead, majority of the research on microalgae as feed material has been focused on monogastric animals and alteration of milk fatty acid (FA) profile of ruminants utilising microalgae supplements high in lipids. *Spirulina platensis* (cyanobacterium) and *Chlorella vulgaris* (chlorophyta), two of the most studied, widely used and commercially available microalgae species, are lower in histidine, but higher in methionine (Mišurçova et al., 2014) than rapeseed meal (Heuzé et al., 2018b; Luke, 2018) and soya bean meal (Heuzé et al., 2017; Luke, 2018). The *in vitro* protein degradability of different microalgae has been very variable and in *S. platensis* it was reported to be higher than for rapeseed meal and soya bean meal (Costa et al., 2016). This is unadvantageous because feeding diets with higher proportion of RDP shifts N metabolism towards unproductive purposes, i.e. increased ruminal ammonia production and urea secretion in milk (Mutswangwa et al., 2016). The variability in chemical composition of different microalgae may also affect the milk production response induced when fed to dairy cows.

Large doses of microalgae in the diet have resulted in feed intake problems on sheep (Hintz et al., 1966) and beef cattle (Van Emon et al., 2015). This is notable because DMI is typically the main determinant affecting milk yield of dairy cows (Hristov et al., 2004). The poor feed intake of microalgae diets has been previously suggested to be linked to dry nature of microalgae (Van Emon et al., 2015). However, the palatability of the diet can probably be affected by feeding technique, as feeding diets as TMR has been reported to decrease feed sorting behaviour of animals which has led to more balanced nutrient intake (DeVries and von Keyserlingk, 2009; Greter et al., 2010). For above-mentioned reasons, the milk production response of microalgae might be lower than that of rapeseed meal. However, microalgae may perform more favourably in comparison to grain legumes, given that rapeseed generally induces greater milk production response than that of soya beans (Huhtanen et al., 2011; Martineau et al., 2013) and faba beans (Puhakka et al., 2016; Ramin et al., 2017) on grass silage-based diets.

2 OBJECTIVES AND HYPOTHESES OF THE STUDY

The aim of the studies reported in this thesis was to evaluate the milk production response and the sustainability of different non-defatted microalgae as protein feeds in the nutrition of lactating dairy cows. This was done in comparison to conventional protein feeds (rapeseed, soya bean meal and faba beans). The sustainability criterion chosen were nitrogen utilisation in milk production (NUE, excretion of N to environment and human-edible protein efficiency), and the sufficient supply and balance of energy and protein. These were evaluated on the grounds of ruminal fermentation and microbial protein production, amino acid metabolism, plasma concentrations of energy metabolites, milk protein yield, milk urea concentration, and excretion of nitrogen in urine and faeces (Figure 3). The microalgae diets constituted of *S. platensis* (I-III), *C. vulgaris* (II), mixture of *S. platensis* and *C. vulgaris* (I), and mixture of *C. vulgaris* and *Nannochloropsis gaditana* (II). Microalgal protein substituted protein in rapeseed meal partially (I, III) or completely (I), soybean meal completely (II), and faba beans partially (III). In addition, the effect of protein supplementation in comparison to unsupplemented diet (I) was studied. The responses of supplemental protein feeding or the source of protein feed on rumen fermentation (I, III), milk production (I-III), N utilisation (I-III) and AA metabolism (I-III) of lactating dairy cows were measured.

The main hypotheses tested in this research were:

- Supplemental protein feeding increases DMI and milk production but decreases conversion efficiency of feed N into milk protein (I).
- The substitution of conventional protein feeds (rapeseed meal, soya bean meal and faba beans) with microalgae decreases DMI (I-II), but this may be avoided with TMR feeding (III).
- Milk production response to microalgae differs depending on the characteristics of microalgae species and substituted conventional protein feed in the basal diet (I-III).
- The N utilisation in milk production is affected by the dietary source of protein, however, the ranking of protein feeds may differ between different N metrics (NUE, excretion of N to environment and human-edible protein efficiency) (I-III).
- The substitution of rapeseed meal with microalgae decreases histidine intake (I, III), and the substitution of grain legumes with microalgae increases methionine intake (II, III). This induces corresponding changes in arterial concentrations of these AA and their metabolites.

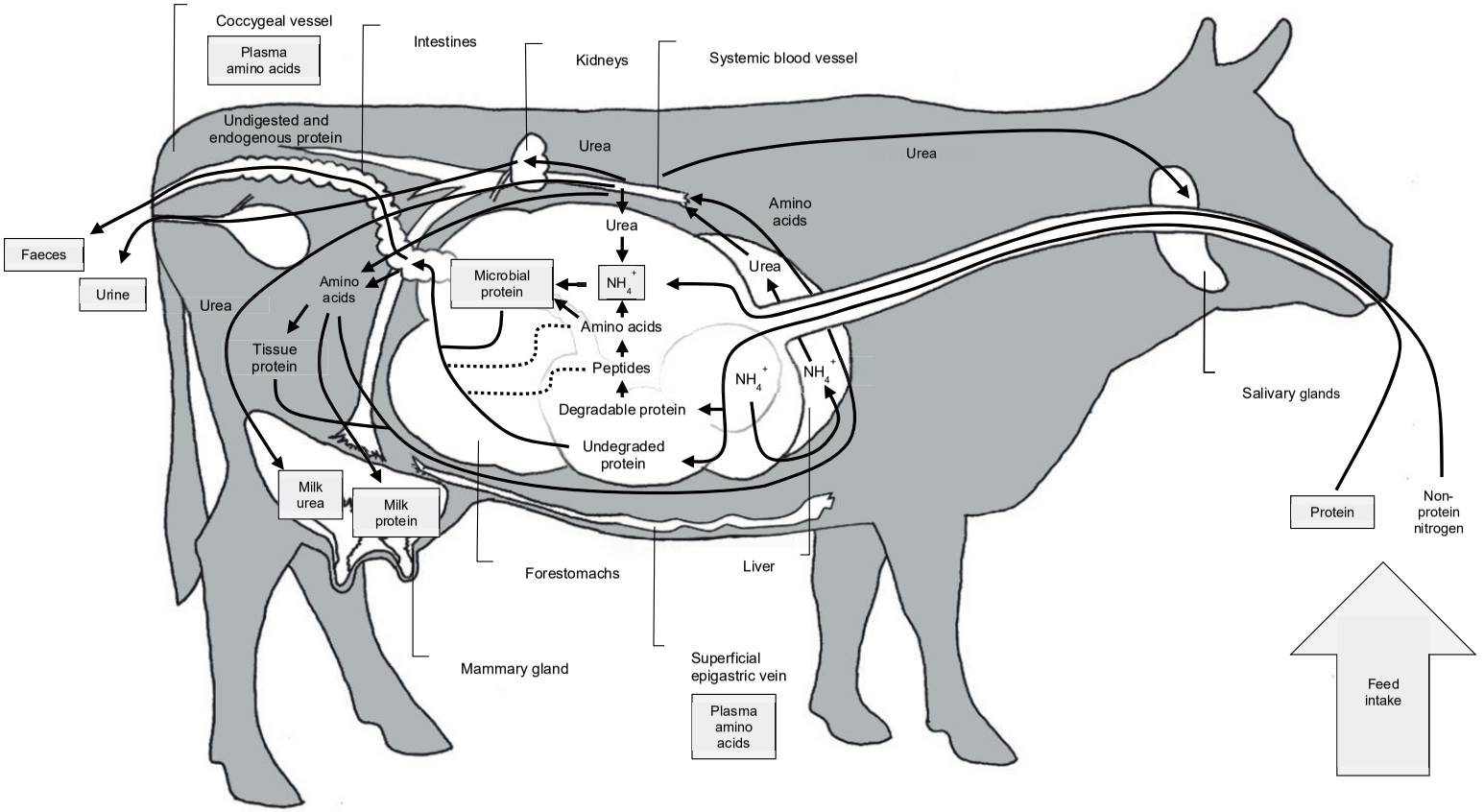


Figure 3 Nitrogen metabolism of lactating cow. Light grey boxes and big grey arrow: measurements related to nitrogen metabolism reported in publications I to III. Rumen NH_4-N was not reported in publication II.

3 SUMMARY OF MATERIALS AND METHODS

The studies documented in publications I-III were carried out as four separate experiments. The experiments were conducted at the University of Helsinki Viikki research farm in Helsinki, Finland. The experimental procedures, chemical analyses, formulas used in calculations and statistical analysis are described in detail in the original publications (I-III). Therefore, only brief outline of the main design, analysis and the measured parameters of each experiment is presented in this section and summarised in Table 2. All experimental procedures were approved by the National Animal Experiment Board in Finland according to the guidelines imposed by the EU Directive 2010/63/EU and the current Finnish legislation on animal experimentation (Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013).

3.1 NOMENCLATURE

In this thesis, cyanobacterium *S. platensis* and *Arhrospira platensis* are considered as synonyms to each other as indicated by Guiry and Guiry (2018). The name *S. platensis* was chosen over *A. platensis* as the microalgae was provided by the supplier under this name. The term microalgae is used in its broadest sense to describe both prokaryotic and eukaryotic microphytes. The microalgae studied in this thesis (*S. platensis*, *C. vulgaris* and *N. gaditana*) belong to phyla *Cyanophyceae*, *Chlorophyta* and *Ochrophyta*, respectively.

The nomenclature of International Union of Pure and Applied Chemistry (IUPAC) was utilised when naming AA to avoid confusion related especially in the naming of methylated histidine molecules. Terms Nπ (nitrogen atom closest to the side chain) and Nτ (nitrogen atom furthest from the side chain) are used later on to describe the position of methylated nitrogen atoms in the imidazole ring of histidine, according to the IUPAC recommendations. This means that the product of muscle actin and myosin catabolism is called Nτ-methylhistidine (traditionally named as 3-methylhistidine in the field of animal science) and the product of anserine breakdown Nπ-methylhistidine (traditionally named as 1-methylhistidine).

3.2 EXPERIMENTAL ANIMALS, TREATMENTS AND PROCEDURES

All experiments were conducted with multiparous Finnish Ayrshire cows in mid- or late lactation (Table 2). The decision to conduct the two first

experiments with late lactating dairy cows was justified by precautionary principle and 3Rs (replacement, reduction and refinement) in animal experimentation to minimise any potential pain, suffering or distress of animals, and to enhance animal welfare. This approach was chosen because prior knowledge about the feed use of microalgae with high inclusion rates for lactating dairy cows was very limited. The experimental design was replicated 3×3 (Exp. 1), replicated and balanced 4×4 (Exp. 2 and 4), and balanced 4×4 (Exp. 3) Latin square (Table 2). Latin square was chosen over continuous design to minimise the number of animals needed without sacrificing the statistical power of the experiments in agreement with 3R principle, and to optimise the amount of other resources needed in the experiments. Change-over designs like Latin squares have been found to be more precise and as accurate as continuous designs in dairy cow experiments when expected differences between treatments are relatively small, and separate feeding of concentrates (fixed rate) and forage (*ad libitum*) is used (Huhtanen and Hetta, 2012). However, the choice between change-over and continuous design may affect the inferred magnitude of milk production response and feed conversion efficiency, but not DMI, milk protein and fat yield and NUE, when dietary CP concentration varies between treatments (Zanton, 2016).

The animals in one block in Experiments 2 and 4 were fitted with rumen cannulae to allow ruminal sampling. The duration of experimental periods was 21d, of which the last 7d formed a sampling period. In the first week of all experimental periods, animals on microalgae diets were accustomed to these feeds by gradually increasing microalgae dose. Animals were housed in individual tie stalls equipped with Roughage Intake Control system (Insentec BV, Marknesse, the Netherlands) and separate concentrate troughs. The latter were used to deliver 11, 12, and 12.5 kg/d of concentrates on fresh matter basis in Experiments 1, 2 and 3, respectively. In Experiment 4, cows were offered TMR with concentrate:forage ratio of 43:57 in DM. Animals had *ad libitum* access to water (all experiments), grass silage (Exp. 1 to 3) and TMR (Exp. 4), and they were milked twice daily.

Experiment 1 examined the effects of partial or complete substitution of rapeseed meal with mixture of *S. platensis* and *C. vulgaris* microalgae on feed intake, milk production, N utilisation and energy and AA metabolism of dairy cows in late lactation. Rapeseed supplement used in this experiment contained 767 g/kg of rapeseed meal and 75 g/kg of turnip rape (*Brassica rapa* subsp. *oleifera*) expeller, the protein of which was isonitrogenously substituted in half or totally with protein in microalgae powder.

In Experiment 2, the effects of protein supplementation, and partial or complete substitution of rapeseed meal with *S. platensis* on feed intake, milk production, rumen fermentation, N utilisation and energy and AA metabolism was studied on dairy cows in late lactation. Rapeseed supplement used in this experiment contained 695 g/kg of rapeseed meal and 138 g/kg of turnip rape expeller, the protein of which was isonitrogenously substituted in half or totally with protein in microalgae powder.

Experiment 3 was conducted to evaluate the responses of feed intake, milk production, N utilisation, energy and AA metabolism, and milk FA composition to different microalgae species supplemented diets in relation to soya bean meal supplemented diet. Mid-lactation cows were used. Soya bean supplement contained 833 g/kg of soya bean meal, the protein of which was isonitrogenously substituted totally with protein in microalgae powder. The focus of this thesis is confined to N utilisation, therefore the results of milk FA composition are mostly excluded from the thesis summary.

Experiment 4 investigated the effects of rapeseed meal or faba beans as sole protein feed or partially substituted with *S. platensis* on feed intake, milk production, N utilisation, and energy and AA metabolism of dairy cows in mid-lactation. The isoenergetic diets were isonitrogenous in regards to N supply from protein feed. In this thesis summary, the focus is on the interactions of substituting conventional protein feeds with microalgae.

All studied microalgae were food-grade originally produced for human consumption. In all experiments, the isonitrogenous protein supplementation, and in Exp. 4 the fixed concentrate:forage ratio in the diets was achieved with adjusting the inclusion rate of cereal-based concentrates. The difference of the smallest and largest daily dose of cereal-based concentrate between all treatments was 1.16 kg DM/d in Exp. 1 (I), 2.63 kg DM/d in Exp. 2 (I), 0.55 kg DM/d in Exp. 3 (II) and 43 g/kg DM in Exp. 4 (III). In Exp. 2, this difference was 1.3 kg DM/d between protein supplemented diets. Because microalgal protein substituted for the protein of rapeseed and turnip rape, or soya bean in all experiments, terms rapeseed meal and soya bean meal are used in the discussion of animal production responses in this thesis, even when these feeds contained molassed sugar beet pulp and molasses as minor components [rapeseed supplement in Exp. 1 and 2 (I), soya bean supplement in Exp. 3 (II)]. Instead, when discussing the nutritive composition of different feeds, terms rapeseed or soya bean meal, and rapeseed or soya bean supplement are used separately, because the contribution of other minor components to the composition of these feeds cannot be separated.

In all experiments, feed intake was determined as the difference between the amount of feed offered and feed refused (I-III). Rumen fermentation was measured by sampling rumen fluid through rumen cannulae at regular intervals (I, III). Faeces and urine were collected as spot samples obtained directly from rectum and vulva, respectively. The total apparent digestibility of the diets and daily faecal volume were determined indirectly by using acid-insoluble ash (AIA) as an internal marker (I-III). Microbial protein production in the rumen and daily urine volume were estimated indirectly based on excretion of urine purine derivatives (I-III). Blood samples taken from superficial epigastric (mammary) vein were considered to represent venous blood (I-III), and that from coccygeal (tail) vessels to represent arterial blood (I-III). Mammary plasma flow was estimated according to the Fick principle based on the stoichiometric transfer of mammary phenylalanine and tyrosine uptake into milk (I-III). Milk yield of all experimental animals was recorded

daily throughout the experiments (I-III). Milk samples were collected for the analysis of fat, protein, lactose and urea (I-III). The mammary metabolism of nutrients was examined by the measurement of arterio-venous differences (I-III).

The studied microalgae were purchased from commercial supplier (Duplaco B.V., Hengelo, the Netherlands), therefore only limited information was available on the production and cultivation conditions of microalgae. *S. platensis* was produced in China in open raceway ponds and *C. vulgaris* was produced in South Korea in closed photobioreactors. After harvesting, both were centrifuged to increase DM content before drying in a dry tower. *N. gaditana* was produced in the Netherlands in open raceway ponds, and centrifuged and dried with drum dryer after harvesting. No information was available on e.g. microalgal strains, nutrient concentration of the growing media, light intensity, temperature and the length of photoperiods in microalgal production systems.

Table 2 The overview of experiments and publications.

Publ.	Exp.	Study design [1]	Animals	Dietary ingredients	Treatments and the dosages of protein feeds
I	1	Replicated 3×3 Latin square	6 multiparous Finnish Ayrshire cows, *n* = 18 DIM 212 ± 30.7 MY 24.8 ± 2.56 kg/d BW 666 ± 53.7 kg	Separate feeding; Grass silage (1st cut), cereal-sugar beet pulp, mineral-vitamin supplement, experimental protein feed	a) Rapeseed supplement (RSS), 2.00 kg DM/d [2] b) Mixture of *Spirulina platensis* and *Chlorella vulgaris* (1:1 on DM basis) (ALG), 0.94 kg DM/d [2] c) Mixture of RSS and ALG (1:1 on CP basis) (RSS-ALG), 1.47 kg DM/d [2]
I	2	Replicated, balanced 4×4 Latin square	8 multiparous Finnish Ayrshire cows, *n* = 32 DIM 190 ± 22.6 MY 35.8 ± 3.08 kg/d BW 718 ± 54.4 kg	Separate feeding; Grass silage (2nd cut), cereal-sugar beet pulp, molassed sugar beet pulp, molasses, mineral-vitamin supplement, experimental protein feed	a) No protein feed (NEG) b) Rapeseed supplement (RSS), 2.55 kg DM/d [2] c) *Spirulina platensis* (ALG), 1.13 kg DM/d [2] d) Mixture of RSS and ALG (1:1 on CP basis) (RSS-ALG), 1.85 kg DM/d [2]
II	3	Balanced 4×4 Latin square	4 multiparous Finnish Ayrshire cows, *n* = 14[3] DIM 112 ± 21.6 MY 36.2 ± 3.77 kg/d BW 652 ± 79.5 kg	Separate feeding; Grass silage (2nd cut), cereal-sugar beet pulp, molassed sugar beet pulp, molasses, mineral-vitamin supplement, experimental protein feed	a) Soya bean supplement (SOY), 1.85 kg DM/d [2] b) *Spirulina platen*sis (SPI), 1.12 kg DM/d [2] c) *Chlorella vulgaris* (CHL), 1.35 kg DM/d [2] d) Mixture of CHL and *Nannochloropsis gaditana* (1:1 on DM basis) (CHL-NAN), 1.63 kg DM/d [2]
III	4	Replicated, balanced 4×4 Latin square; 2×2 factorial arrangement of treatments	8 multiparous Finnish Ayrshire cows, *n* = 32 DIM 113 ± 36.3 MY 33.9 ± 4.79 kg/d BW 707 ± 63.2 kg	Total mixed ration; Grass silage (2nd cut), barley, sugar beet pulp, mineral-vitamin supplement, experimental protein feed	a) Rapeseed meal (RSM), 95.0 g/kg DM [2] b) Mixture of RSM and *Spirulina platensis* (1:1 on CP basis) (RSM-SPI), 73.9 g/kg DM [2] c) Faba beans (FB), 117 g/kg DM [2] d) Mixture of FB and *Spirulina platensis* (1:1 on CP basis) (FB-SPI), 85.2 g/kg DM [2]

n = number of experimental units used in the statistical analysis, DIM = days in milk at the beginning of exp., MY = milk yield at the beginning of exp., BW = body weight at the beginning of exp., DM = dry matter, CP = crude protein.
[1] In all experiments, the duration of experimental periods was 21d, of which the last 7d formed a sampling period.
[2] Isonitrogenous supply from protein feed.
[3] Two missing observations in Exp. 3, one on CHL (period 3) and the other on SPI (period 4).

4 RESULTS AND DISCUSSION

4.1 CHEMICAL COMPOSITION OF MICROALGAE

The chemical composition of microalgae is highly variable depending on species and strain. Some microalgae species (such as *S. platensis*; Becker, 2014) are very rich in CP, whereas others mainly accumulate lipids (such as *Schizochytrium* sp.; Bernaerts et al., 2018) or carbohydrates (e.g. *Porphyridium cruentum*; Becker, 2014). The chemical composition of *S. platensis* and *C. vulgaris* used in Experiments 1 to 4 (I-III) showed only little variation between experiments. This was unexpected given the huge differences in composition within species reported in literature (Table 3). However, it may be attributed to the fact that the producers of the studied microalgae were the same throughout the experiments, thus the production conditions were probably relatively similar. Indeed, the chemical composition of microalgae can be manipulated e.g. with the alteration of nutrient concentration in growth medium. Lipid (Hu et al., 2008), protein (Fernandes et al., 2016) and starch concentration (Brányiková et al., 2011), and AA (Templeton and Laurens, 2015) and FA composition (Olmstead et al., 2013) of microalgae are only some examples of nutrients whose quantity and composition can be greatly affected by growing conditions. This modifiability of the chemical composition of microalgae enables multiple different applications for the same species, and optimisation of the biomass composition for different purposes. Moreover, due to the highly variable composition of microalgae, it is a necessity to always specify the species studied and preferably also the strain, growing conditions and harvesting method of algae, if possible. This allows better replicability of experiments focusing on both production and utilisation of algae.

4.1.1 NITROGEN AND AMINO ACIDS

In agreement with literature, *S. platensis* and *C. vulgaris* used in current experiments were very high in N compared to conventional protein feeds such as rapeseed meal and soya bean meal (Table 3). In contrast, the N concentration of *N. gaditana* used in Experiment 3 (II) was lower than that of other two studied microalgae and closer to that of conventional protein feeds. Table 3 includes own results for rapeseed meal and faba beans in Exp. 4 (III) only, because in Exp. 1 and 2 (I) rapeseed supplement and in Exp. 3 (II) soya bean supplement were used, both containing molassed sugar beet pulp and molasses as minor components.

Of essential AA (EAA), the methionine concentration of microalgae (18.3 to 22.9 g/kg CP) was higher or comparable to that of rapeseed supplement and

Table 3 Average chemical composition (g/kg DM) of different non-defatted microalgae species and conventional protein feeds in current experiments (I-III) and literature.

	Organic matter	Nitrogen	Crude fat	NDF[1]	iNDF[2]	Starch	Reference
Microalgae							
Spirulina platensis	*n* = 4[3]	*n* = 4	*n* = 4	*n* = 2			Panjaitan et al., 2015; Costa et al., 2016; Matos et al., 2016; Cuellar-Bermúdez et al., 2017
Own result (I-III): Mean ± SD	930 ± 2.2	110 ± 0.99	54.3 ± 3.80	0 ± 0	NA	57.1 ± 15.0	
Literature: Mean ± SD	879 ± 44.2	105 ± 7.98	70.9 ± 47.3	49.0 ± 19.8	NA	NA	
Literature: Min - Max	816 - 912	96.8 - 114	8.40 - 114	35.0 – 63.0			
Chlorella vulgaris	*n* = 6	*n* = 7	*n* = 7	*n* = 2			Matos et al., 2016; Kholif et al., 2017; Adamakis et al., 2018; Bernaerts et al., 2018; Tsiplakou et al., 2018
Own result (I-II): Mean ± SD	946 ± 4.2	95.5 ± 2.5	109 ±19.7	0 ± 0	NA	48.5 ± 7.85	
Literature: Mean ± SD	804 ± 77.4	72.0 ± 25.7	155 ± 115	110 ± 25.5	NA	NA	
Literature: Min - Max	713 - 905	41.5 - 108	10.5 - 366	92.0- 128			
Nannochloropsis gaditana	*n* = 2	*n* = 7	*n* = 7				Sudasinghe et al., 2015; Fernandes et al., 2016; Matos et al., 2016
Own result (II): Mean	842	61.6	192	90.0	NA	26.1	
Literature: Mean ± SD	900 ± 42.3	28.5 ± 30.0	67.1 ± 70.3	NA	NA	NA	
Literature: Min - Max	870 - 930	4.51 – 91.9	15.4 - 217				
Conventional protein feeds							
Rapeseed meal	*n* = 13	*n* = 20	*n* = 19	*n* = 20	*n* = 3	*n* = 3	Bell and Keith, 1991; Tesfa et al., 1995; Rinne et al., 1999; Kokkonen et al., 2000, 2002; Getachew et al. 2004; Huuskonen, 2009; Krizsan and Huhtanen, 2013; Maxin et al., 2013a
Own result (III): Mean	916	55.2	41.1	318	167	32.0	
Literature: Mean ± SD	919 ± 4.06	61.8 ± 9.98	45.9 ± 14.1	269 ± 29.6	103 ± 10.3	24.7 ± 8.08	
Literature: Min - Max	912 - 927	24.6 - 70.1	22.7 – 78.0	226 - 319	94.0 - 114	16.0 - 32.0	
Soya bean meal	*n* = 11	*n* = 11	*n* = 10	*n* = 10	*n* = 3	*n* = 6	Lekule et al., 1990; Getachew et al., 2004; Krizsan and Huhtanen, 2013; Gidlund et al., 2015; de Almeida et al., 2018
Literature: Mean ± SD	933 ± 22.2	78.5 ± 7.86	27.0 ± 8.59	162 ± 44.3	5.63 ± 3.46	28.7 ± 12.6	
Literature: Min - Max	902 - 993	57.1 - 86.6	13.6 - 40.0	96.0 - 237	2.00 - 8.90	17.0 - 49.0	
Faba beans	*n* = 6	*n* = 30	*n* = 30	*n* = 14	*n* = 2	*n* = 30	Bhatty, 1974; Duc et al., 1999; Ramin et al., 2017; Nalle et al., 2010
Own result (III): Mean	957	49.6	15.0	158	3.22	346	
Literature: Mean ± SD	963 ± 4.9	48.2 ± 3.99	14.8 ± 5.30	180 ± 23.2	3.21 ± 0.01	390 ± 43.1	
Literature: Min - Max	957 - 967	36.6 - 55.5	7.00 - 23.7	134 - 221	3.20 - 3.22	271 – 444	

n = NA = not available.

[1] Own results analysed using crucibles with pore size of 40 to 100 µm. In Experiment 3 (III), microalgae were also analysed using crucibles with pore size of 16 to 40 µm. This resulted in NDF concentrations of 87.4, 15.1 and 219 g/kg DM for *S. platensis*, *C. vulgaris* and *N. gaditana*, respectively.

[2] Own results analysed with *in situ* rumen incubation method using nylon bags with mesh size of 17 µm.

[3] Number of observations for literature values

meal (12.0 to 20.4 g/kg CP), and higher in comparison to soya bean supplement (9.76 g/kg CP) and faba beans (5.22 g/kg CP) (Figure 4). This indicates that microalgae might be suitable protein source complementing leguminous foods and feeds that are typically low in methionine (Luke, 2018). Generally, microalgae are poorer sources of histidine (14.7 to 18.3 g/kg CP) than conventional protein feeds (21.3 to 28.0 g/kg CP in rapeseed, soya bean and faba beans; Figure 4), which is noteworthy on cereal and grass silage-based ruminant diets that are typically deficient on histidine (Kim et al., 1999; Vanhatalo et al., 1999). However, contradicting AA profiles for microalgae have sometimes been reported, e.g. Safi et al. (2013) with methionine concentration of 9.0 and 11.6 g/kg CP and histidine concentration of 17.2 and 6 g/kg CP for *S. platensis* and *C. vulgaris*, respectively. Due to the presence of other minor feed components, the concentration of methionine was slightly lower for soya bean supplement (II) than for soya bean meal (Heuzé et al., 2017), and the concentration of methionine slightly lower and histidine slightly higher for rapeseed supplement (I) than for rapeseed meal (III).

Instead of the CP concentration, the N concentration of different microalgae and conventional protein feeds is reported in Table 3 to allow fair comparison between different feed materials. The majority of protein analysis methods measure elemental N concentration of feed material, which is then converted to CP concentration by N-to-protein conversion factor. According to the AOAC Official method 2001.11 for analysis of Kjeldahl-N, the conversion factor 6.25 is generally used in majority of feeds, excluding that of 5.70 for wheat and 6.38 for dairy products (Thiex et al., 2002). The conversion factor of 6.25 assumes that N is derived from protein containing 160 g N/kg (1000/160=6.25) and gives an approximate protein value (CP) (McDonald et al., 2002). In addition to true protein, CP includes NPN such as free amino acids, amines and nucleic acids.

No universally agreed conversion factor exists for microalgae, but conversion factors of 2.53 to 5.77 (Lourenço et al., 2004), 4.68 to 5.35 (Templeton and Laurens, 2015), 5.95 (López et al., 2010), and 6.25 to 6.35 (Safi et al., 2013) have been suggested for various species. The differences in microalgal N-to-protein conversion factors is caused by the concentration of many NPN compounds present in microalgae, such as chlorophyll, nucleic acids and amino sugars (Templeton and Laurens, 2015). Lourenço et al. (2004) reported that NPN accounted for 2 to 35% of the total N in microalgae. This is also one of the reasons why microalgae may be recommended only as minor feed component for pigs and poultry, which tolerate NPN more poorly than ruminants (Patra and Aschenbach, 2018). The A fraction of Cornell Net Carbohydrate and Protein System, which constitutes of NPN, has been reported to be 4.9 to 11.7 % of total N in rapeseed meal (Ahvenjärvi et al., 1999; Choi et al., 2002, 2003; Chrenková et al., 2014), 2.8 to 15.5 % of total N in soya bean meal (Fortina et al., 2003; Chrenková et al., 2014) and 12.9 % of total N in faba beans (Chrenková et al., 2014).

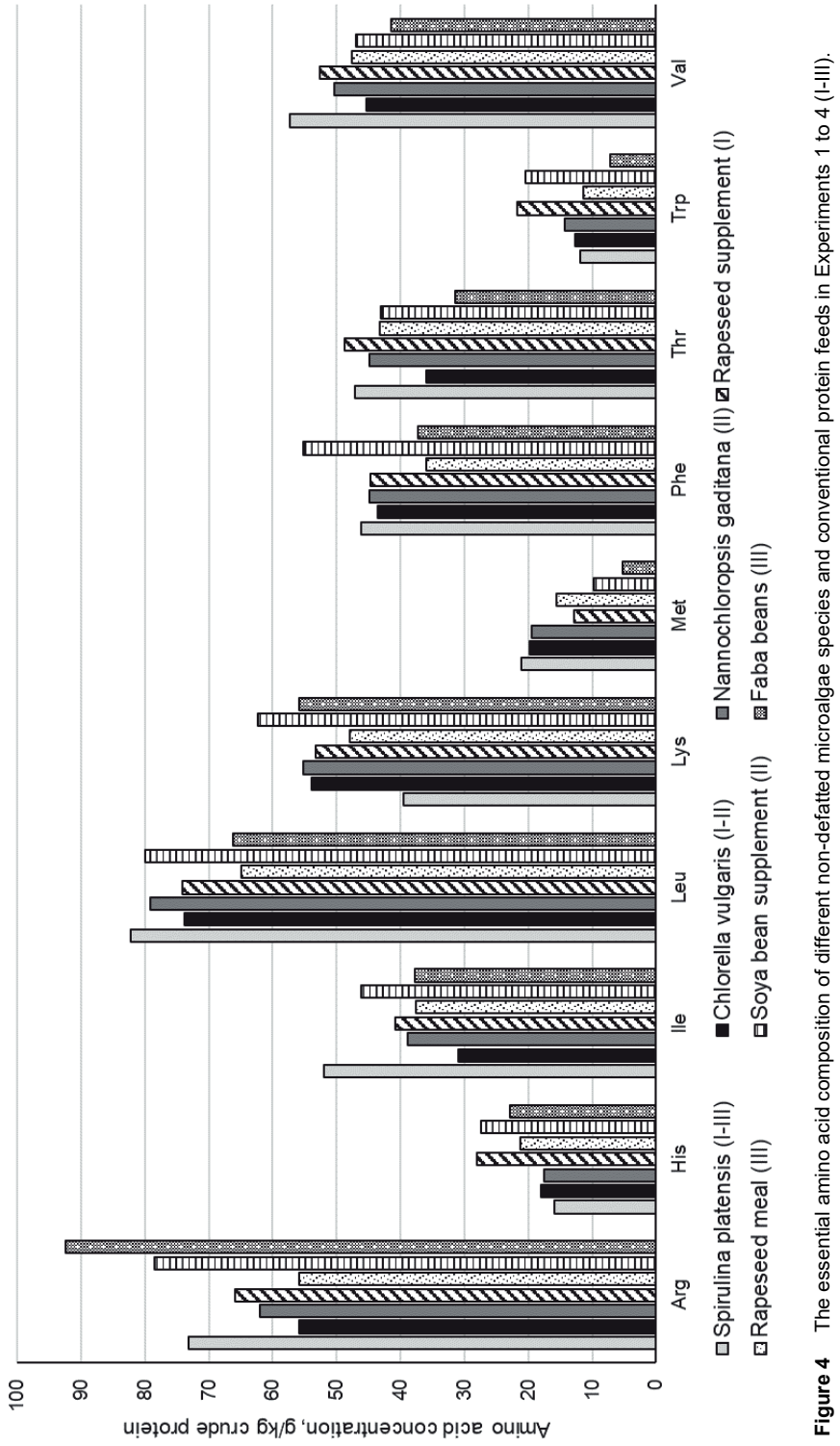


Figure 4 The essential amino acid composition of different non-defatted microalgae species and conventional protein feeds in Experiments 1 to 4 (I-III).

However, when comparing the N concentration calculated based on AA composition and Kjeldahl-N concentration, the NPN concentration of microalgae did not seem to differ from conventional feeds (Table 4). Moreover,

Table 4. The nitrogen (N) concentration (g N/kg DM) of feeds in Experiments 1 to 4 as calculated based on amino acid (AA) composition and determination by Kjeldahl method

				AA-N:Kjeldahl-N	
	Exp.	**AA-N[1]**	**Kjeldahl-N**	**Own result**	**Literature values[2]**
Microalgae					
Spirulina platensis	1	96.9	110	0.882	
	2	102	112	0.916	
	3	106	112	0.944	
	4	100	109	0.919	
Chlorella vulgaris	1	71.7	97.3	0.747	
	3	82.1	93.7	0.876	
Nannochloropsis gaditana	3	55.6	61.6	0.903	
Conventional feeds					
Rapeseed supplement	1	43.0	51.2	0.839	
	2	47.6	49.7	0.958	
Rapeseed meal	4	44.2	55.3	0.800	0.862 - 0.910 Choi et al., 2003; Messerschmidt et al., 2014; Kaewtapee et al., 2018
Soya bean supplement	3	70.8	70.2	1.01	Soya bean meal 0.935 - 0.957 Burr et al. 2011; Dilger et al., 2014; Kaewtapee et al., 2018
Faba beans	4	44.0	49.6	0.887	0.837 - 1.04 Duc et al., 1999
Cereal-sugar beet pulp	1	16.4	20.8	0.786	
	2	16.7	19.1	0.873	
	3	17.2	19.8	0.867	
Barley	4	16.1	19.4	0.832	0.843 – 1.01 Choi et al., 2003; Bachmann et al., 2018
Molassed sugar beet pulp	2	13.4	18.1	0.739	
	3	13.4	17.8	0.754	
Molasses	2	4.76	16.9	0.282	
	3	3.31	11.0	0.313	
Grass silage	1	13.7	22.0	0.623	0.668 - 0.838
	2	17.1	21.3	0.800	Choi et al., 2003;
	3	16.0	21.6	0.741	Halmemies-Beauchet-
	4	14.2	25.4	0.565	Filleau et al., 2014

[1] In the calculation of own and literature data, the molecular weight of one H_2O molecule extracted in the formation of amide bonds is taken into account. Results were calculated based on the N concentration of the following AA residues (g/g AA residue): 0.359 for arginine, 0.306 for histidine, 0.124 for isoleucine, 0.124 for leucine, 0.219 for lysine, 0.107 for methionine, 0.095 for phenylalanine, 0.139 for threonine, 0.150 for tryptophan, 0.141 for valine, 0.197 for alanine, 0.122 for aspartic acid, 0.136 for cysteine, 0.109 for glutamic acid, 0.246 for glycine, 0.144 for proline, 0.161 for serine and 0.086 for tyrosine.

[2] Lacking AA-N values: Choi et al., 2003 (tryptophan), Burr et al., 2011 (cysteine, tryptophan), Halmemies-Beauchet-Filleau et al., 2014 (tryptophan); Messerschmidt et al., 2014 (tyrosine), Kaewtapee et al., 2017 (tyrosine).

in most cases the values obtained for conventional feeds were in agreement with literature. The ratio of AA-N:Kjeldahl-N was on average 0.90 for microalgae, 0.85 for other concentrates excluding molasses, and 0.70 for silage. Therefore, the use of the same N-to-protein conversion factor (6.25) for all experimental feeds was considered justified. However, it should be noted that in current experiments the calculation of AA-N was based only on 18 of the 21 (eukaryotes) or 22 (prokaryotes) proteinogenic AA (EAA and alanine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and tyrosine). The concentration of all these AA was not always available on the literature (for further information, see footnotes of Table 4). The low ratio for silage is logical given the NH_4-N and other NPN components present in preserved forage.

4.1.2 FIBRE FRACTIONS

According to own and other published records, the neutral detergent fibre (NDF) concentration of microalgae is variable but generally lower than in conventional protein feeds (Table 3). However, the analysis of fibrous components in unicellular microalgae with very small particle size is challenging with standard methods designed to feed components with larger particle size. Zero NDF concentrations of *S. platensis* and *C. vulgaris* were consistently measured in current experiments (I-III) using crucibles with pore size of 40 to 100 µm. When crucibles with smaller pore size (16 to 40 µm) were used in Experiment 3 (II), NDF results of 87.4, 15.1 and 219 g/kg DM were obtained for *S. platensis*, *C. vulgaris* and *N. gaditana*, respectively. On the contrary, when samples of *S. platensis* and *C. vulgaris* were placed in nylon bags with mesh size of 17 µm and washed with pure tap water, no residues of samples were left in these bags in our laboratory. Based on this observation, the indigestible NDF (iNDF) determination of microalgae was considered impossible with *in situ* rumen incubation method. Indeed, at least some part of the variation of NDF concentration in microalgae reported in research literature is likely attributable to the diversity in analytical methods. For the analysis of NDF, crucibles with pore size of 40 to 60 µm were used in Costa et al. (2016) (P. Isherwood, personal communication, 2 November 2016), and Ankom fibre bags with porosity of 25 µm in Kholif et al. (2017) (A. Kholiff, personal communication, 23 July 2018). Both Costa et al. (2016) and Kholif et al. (2017) obtained results for NDF concentration of *S. platensis* (63 g/kg DM) and *C. vulgaris* (92 g/kg DM), respectively. Griffiths et al. (2012) reported that the particle size of *S. platensis* was 10 × 60 to 500 µm (filamentous cell shape), that of *C. vulgaris* 2.5 µm in diameter (spherical cell shape), and that of *Nannochloropsis* sp. 3.0 µm in diameter (spherical cell shape). Given this small particle size, it is remarkable that any NDF at all were detected with standard methods. The difficulty of fibre analysis of microalgae was further demonstrated by Costa et al. (2016), who reported negligible and illogically higher acid detergent fibre (ADF) than NDF concentrations for some

microalgae species. Considering these challenges when analysing microalgal fibrous components, further development of the fibre analysis methods are needed, e.g. with addition of a microfiber filtering aid having a very small pore size (1.5 μm) (Raffrenato and Van Amburgh, 2011). Moreover, the reporting of pore sizes of the apparatus used in the fibre analysis can be considered essential for the replicability of the results.

The main component of *S. platensis* cell wall is murein (peptidoglycan), which is a polymer composed of *N*-acetylglucosamine, *N*-acetylmuramic acid and several different AA (Lee, 2008). Polysaccharides are likely found only in minor concentrations in cell walls of *S. platensis*, and glucose (49.8 %), mannose (29.8 %), rhamnose (6.7 %) and galacturonic acid (5.6 %) monosaccharides have been reported to be their main constituents (Bernaerts et al., 2018). These monosaccharides could form many different polymers such as cellulose, glucomannan (hemicellulosic polysaccharide) or rhamnogalacturonans (pectic polysaccharide). However, as long as the exact polymeric structure of cell wall components of *S. platensis* remains unknown, only little can be deduced about the fiber composition of this microalgae. The same applies to many other microalgae, too. Costa et al. (2016) reported NDF and ADF concentrations of 63 and 0 g/kg DM, respectively, for *S. platensis*. This suggests that the fibrous components present in *S. platensis* could mainly compromise of hemicelluloses. Some, but not all, cyanobacteria species may have cellulose as minor extracellular components in slime tubes, sheaths and extracellular slime (Nobles et al., 2001). According to Bernaerts et al. (2018), extracellular polysaccharides make up 0.7 % of the dry weight of *S. platensis* and they constitute mainly of rhamnose (29.1 %), ribose (25.8 %), glucose (11.8 %), galactose (11.1 %), fucose (8.9 %) and galacturonic acid (8.9 %).

The cell wall of *C. vulgaris* is commonly reported to be more rigid than that of evolutionary older cyanobacteria (e.g. Mendez et al., 2015). This contradicts our result with lower NDF concentration (obtained on crucibles with smaller pore size) in *C. vulgaris* than in *S. platensis* (cyanobacterium). However, the exact cell wall composition of *C. vulgaris* is poorly understood (Mahdy et al., 2015), and discrepancies regarding the cell wall structure of *Chlorella* sp. in general have been described in research literature (Bernaerts et al., 2018). The cell wall polysaccharides of *C. vulgaris* are mainly constituted of glucose (41.5 %) and mannose (34.8 %) monosaccharides, which suggests the occurrence of glucomannans in the cell walls (Bernaerts et al., 2018). Also the presence of glucosamines has been suggested for *C. vulgaris* (Takeda, 1991). Nevertheless, the digestibility of *C. vulgaris* in anaerobic fermentation seems to be mainly determined by proteinaceous polymers instead of carbohydrates (Mahdy et al., 2015). The NDF concentrations of 92 and 128 g/kg DM (Costa et al., 2016; Tsiplakou et al., 2018, respectively) and ADF concentration of 42 g/kg DM (Tsiplakou et al., 2018) have been reported for *C. vulgaris*. This is strikingly different from our NDF result of 15.1 g/kg DM obtained on crucibles with smaller pore size.

The cell wall of *N. gaditana* has bilayered structure with an outer alganean layer that protects carbohydrate-rich inner wall (Scholz et al., 2014) with glucose as a main polysaccharide (75.8 %) (Bernaerts et al., 2018). Indeed, Scholz et al. (2014) reported that the inner cell wall of *N. gaditana* is mainly comprised of cellulose. The alganean layer in *N. gaditana* closely resembles the cutan of vascular plants and is comprised of long, straight-chain, saturated aliphatic compounds with ether cross-links (Scholz et al., 2014). To the author's knowledge, the NDF concentration of *N. gaditana* has not been reported previously in literature. However, Gatrell et al. (2015) reported the NDF concentration of defatted *N. oceanica* biomass to be 254 g/kg DM, which is in line with the concentration of 219 g/kg DM obtained in Experiment 3 (II) using crucibles with smaller pore size. Irrespective of the fibre concentration and composition in microalgae, the possible microalgal fibre components unlikely have any physically effective properties for rumen function due to their microscopic particle size.

4.1.3 LIPIDS AND FATTY ACIDS

The crude fat concentration of microalgae used in current experiments was relatively low considering the literature values reported for some microalgae species (e.g. *Schizochytrium* sp. with crude fat concentration up to 739 g/kg DM; Bernaerts et al., 2018). Similarly to other nutrients, also microalgal crude fat (Table 3) and lipid composition (Table 5; Yao et al., 2015) are very variable between and within species. According to Yao et al. (2015), the proportion of triacylglycerides may vary from 4 to 78% of all lipids in different microalgae species, free fatty acids from 0.7 to 31%, and glyco- and phospholipids from 0.7 to 25%. Comparably, triacylglycerides and phospholipids make up 92% and 3 to 4%, respectively, of the total lipids in rapeseed (Zadernowski and Sosulski, 1978) and 95 to 97%, and 1.5 to 2.5%, respectively, of the total lipids in crude soya bean oil (Wang, 2011).

The FA profile of *S. platensis* in Experiment 3 (II) with 16:0, *cis*-6, *cis*-9, *cis*-12 18:3 (ɣ-linolenic acid; GLA) and *cis*-9, *cis*-12 18:2 being the three main FA (Table 5) is in line with Jafari et al. (2014). The high concentration of GLA in *S. platensis* is very unique, as vegetable lipids are typically low in this FA (Callaway, 2004). The FA profile of *C. vulgaris* in Experiment 3 (II) comprised mainly of *cis*-9, *cis*-12 18:2, Δ16:2 (location and configuration of double bonds undetermined) and 16:0 (Table 5). This is consistent with Zayadan et al. (2017), who also reported that the 16:2 FA in *C. vulgaris* constituted of *cis*-7, *cis*-10 16:2 (6.2 to 21.0 g/100g FA) and *cis*-9, *cis*-12 16:2 (0.6 to 0.8 g/100g FA). In contrast, very different FA profile of *C. vulgaris* was reported by Khoeyi et al. (2012) with 16:0 (20.2 to 22.1 g/100g FA), *cis*-9, *cis*-12, *cis*-15 18:3 (18.0 to 19.4 g/100g FA) and *cis*-9 18:1 (10.7 to 12.1 g/100g FA) as main FA. Three main FA in *N. gaditana* were *cis*-9 16:1, 16:0 and *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5 (eicosapentaenoic acid, EPA) (II; Table 5). This is in agreement with Mourente et al. (1990), however, Matos et al. (2016) reported

dissimilar FA profile for *N. gaditana* with 16:0 and 16:1 as main FA (53 and 20 g/100g FA, respectively) but no detectable EPA. Typically, the distinctive characteristics of many *Nannochloropsis* species is their relatively high EPA concentration (Mourente et al., 1990). This difference might probably be explained by differences in cultivation conditions of microalgae. Olmstead et al. (2013) reported that EPA concentration of *Nannochloropsis* sp. may vary from 5.7 to 16.6 g/kg depending on N sufficiency in growing medium.

Another explanation for varying FA composition might be also methodological differences. Olmstead et al. (2013) observed that the cultivation conditions also affected the concentrations of FA in different lipid fractions (neutral vs. polar). As the solubility of different lipid fractions into various solvents vary (Shadidi, 2001), relatively different FA profiles for the same microalgae species might be obtained depending on the cultivation conditions of microalgae and solvent used in FA analysis.

Table 5. Fatty acid (FA) composition of different microalgae species and soya bean supplement (Exp. 3), and rapeseed supplement (Exp. 2). Major FA of each feed are marked with bold text.

	Rapeseed suppl.	**Soya bean suppl.**	***Spirulina platensis***	***Chlorella vulgaris***	***Nannochloropsis gaditana***
Total FA, g/kg DM	25.2	9.86	28.6	33.1	28.9
FA composition, g/100g FA					
14:0	0.166	0.129	0.160	0.055	3.00
16:0	**8.27**	**14.7**	**45.7**	**15.8**	**24.3**
cis-9 16:1	0.897	0.163	2.76	0.493	**35.5**
trans-3 16:1			1.20	1.08	1.23
∆16:2				**26.4**	
18:0	2.65	2.95	1.05	0.160	0.719
cis-9 18:1	**43.1**	**20.5**	2.70	2.47	5.38
cis-11 18:1	9.23	2.22	0.622	0.736	0.586
cis-9, *cis*-12 18:2 (n-6)	**25.1**	**50.5**	**23.5**	**48.5**	1.40
cis-9, *cis*-12, *cis*-15 18:3 (n-3)	8.05	7.45	0.372	2.31	0.034
cis-6, *cis*-9, *cis*-12 18:3 (n-6)	0.033		**19.9**	0.033	0.224
cis-8, *cis*-11, *cis*-14 20:3 (n-6)			0.232		1.29
cis-5, *cis*-8, *cis*-11, *cis*-14 20:4 (n-6)			0.044		2.14
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5 (n-3)	0.035			0.021	**19.2**
Σ Unid. FA					2.89
Σ SFA	12.4	18.8	47.7	17.0	29.8
Σ MUFA	54.3	23.3	8.03	5.51	43.0
Σ PUFA	33.3	57.9	44.3	77.5	24.4

Unid. = unidentified; SFA = saturated FA; MUFA = monounsaturated FA; PUFA = polyunsaturated FA.

4.2 FEED INTAKE AND DIGESTIBILITY

4.2.1 FEED INTAKE AND PALATABILITY

Protein supplementation

Protein supplementation did not affect total DMI in Experiment 2 (I). This contradicts the common response of increasing DMI to protein supplementation irrespective of the supplementary protein source and basal diet (e.g. Oldham, 1984; Allen, 2000; Huhtanen et al., 2011). However, the increased DMI with protein supplementation is usually mediated via increased digestibility of especially fibrous fractions allowing increased voluntary feed consumption (Oldham, 1984). Indeed, silage intake tended to increase with protein supplementation in Experiment 2 (I), which is also in agreement with Khalili et al. (2002) and Huhtanen et al. (2008b, 2011). The absence of total DMI response to protein supplementation in Experiment 2 (I) was caused by the negative effect of microalgae on concentrate intake. When comparing only unsupplemented diet and rapeseed meal supplemented diet, there was a numerical increase of 0.57 kg/d in DMI as dietary CP concentration increased 21 g/kg DM from 125 to 146 g/kg DM. This agrees relatively well with the estimated increase of 0.63 kg/d in DMI established from the equations for rapeseed meal supplemented diets in meta-analysis of Huhtanen et al. (2011).

Microalgae vs. conventional protein feeds

The response of DMI to substitution of conventional protein feeds with microalgae depended on the feeding method applied in the experiments. When concentrates and forage were fed to cows separately, the quantity of DMI was not affected by the substitution of rapeseed meal with the mixture of *S. platensis* and *C. vulgaris* (Exp. 1; I), or *S. platensis* (Exp. 2; I), or the substitution of soya bean meal with various microalgae (Exp. 3; II). However, the composition of DMI changed in Experiments 2 (I) and 3 (II), as indicated by decreasing proportion of concentrate in the microalgae diets in comparison to rapeseed meal (Exp. 2; I) and soya bean meal (Exp. 3; II) diets. This was the consequence of incomplete concentrate intake and simultaneously increased silage intake to compensate poorer palatability of microalgae containing concentrate. When cows no longer had the opportunity to avoid microalgae in TMR feeding (Exp. 4; III), the quantity of DMI was decreased when the protein of rapeseed meal and faba beans was substituted in half with *S. platensis*. Plotting DMI data of individual cows by day during the whole Experiment 4 did not indicate any distinctive feed intake pattern (e.g. adaption) on microalgae diets.

All studied microalgae caused decrease in concentrate intake in Experiment 3 (II), however, the response was most pronounced on diet containing mixture of *C. vulgaris* and *N. gaditana*. This diet resulted in 2.2 kg

DM/d higher silage intake and 2.2 kg DM/d lower concentrate intake compared to soya bean meal. Due to the differences in microalgal CP concentration, the daily microalgae dosage was higher on the diet containing mixture of *C. vulgaris* and *N. gaditana* (+0.51 and +0.30 kg DM/d, respectively) than on diets containing *S. platensis* or *C. vulgaris* (Table 6). This might have further emphasised the palatability problems on mixture of *C. vulgaris* and *N. gaditana*. Moreover, there was quite large individual variation in the acceptability of microalgae containing concentrates. While some cows ate the microalgae containing concentrates with relatively similar quantities to control feeds, some cows responded with dramatic drop in concentrate intake and increase in silage intake (Figure 5). The largest changes were observed in Experiment 3 (II), in which the proportion of concentrates in the dietary DM was as low as 26% in one individual animal.

In contrast to other experiments, microalgae inclusion in the diet did not affect the quantity or composition of DMI in Experiment 1 (I). The reason for this difference between Experiment 1 and Experiments 2 to 4 remains unclear, as the experiments did not differ in DMI or amount or proportion of microalgae on diets (Table 6). The cows in Experiment 1 (I) had lower milk yield than cows in other experiments (Table 2), thus the difference in feed intake responses might be related to differences in metabolism or nutrient requirements. In Experiment 1 (I), *S. platensis* and *C. vulgaris* were fed to animals as a mixture. Therefore, it is also possible that there was some kind of synergism between these two microalgae that for some reason increased the palatability of microalgae containing concentrates. In Experiment 3 (II), the least negative effect of microalgae on concentrate palatability was observed on *C. vulgaris*. On this diet, the proportion of concentrate in the diet was only 6.7% lower than on soya bean meal. *S. platensis* and *C. vulgaris* microalgae studied in Experiments 1 to 4 (I-III) were purchased from the same supplier and produced under similar conditions, and their composition was very similar between experiments. Thus, the variability in microalgal composition unlikely explains the differences between Experiment 1 and Experiments 2 to 4.

In general, there are only few microalgae experiments on ruminants with relatively large dosage of microalgae. Moate et al. (2013) did not notice any concentrate intake problems on dairy cows related to algae meal high in crude fat (556 g/kg DM). However, forage intake was decreased as much as 2.9 kg/d when microalgae meal was incorporated into dairy cow diets with dosage of 124 to 373 g/d (Moate et al., 2013). da Silva et al. (2016) noticed no difference in DMI when algae meal (dosage 92 g/kg DM) consisting of 570 g/kg defatted microalgae and 430 g/kg soyhulls substituted 34% for ground maize in dairy cow diets. However, Van Emon et al. (2015) reported palatability problems on beef steers with microalgae diets relatively low in crude fat (71.5 to 75.4 g/kg DM). In their experiments, algae meal consisting of defatted microalgae and soyhulls substituted for maize based concentrates, and was included in the diet

Table 6. The amount and proportion of microalgae on diets in Experiments 1 to 4 (I-III).

Protein feed treatments[1]	DMI, kg/d [2]	Concentrate crude fat, g/kg DM	Planned microalgae dose, kg DM/d	Microalgae eaten, kg DM/d	Microalgae, % of concentrate DM	Microalgae, % of DMI
Exp. 1 (I)						
RSS	21.9	53.4				
RSS-ALG	22.4	54.6	0.47	0.47	4.65	2.10
ALG	22.3	55.8	0.94	0.93	9.31	4.22
Exp. 2 (I)						
NEG	22.8	47.0				
RSS	23.4	45.4				
RSS-ALG	23.0	45.8	0.57	0.54	5.28	2.34
ALG	22.8	46.3	1.13	0.99	10.4	4.36
Exp. 3 (II)						
SOY	21.5	39.2				
SPI	22.0	44.5	1.12	0.90	9.91	4.10
CHL	20.9	53.1	1.35	1.20	12.0	5.72
CHL-NAN	21.6	60.0	1.63	1.26	14.3	5.83
Exp. 4 (III)						
RSM	23.3	34.4[3]				
RSM-SPI	22.8	34.4[3]		0.60		2.64
FB	23.1	31.6[3]				
FB-SPI	22.3	33.1[3]		0.59		2.64

DM = dry matter, DMI = dry matter intake

[1] Exp. 1 and 2: NEG = no protein feed; RSS = rapeseed supplement; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* microalgae (Exp. 1) or *S. platensis* (Exp.2); RSS-ALG = mixture of RSS and ALG. Exp. 3: SOY = soya bean meal; SPI = *S. platensis* microalgae; CHL = *C. vulgaris* microalgae; CHL-NAN = mixture of *C. vulgaris* and *Nannochloropsis gaditana* microalgae. Exp. 4: RSM = rapeseed meal; RSM-SPI = rapeseed meal and *S. platensis* microalgae; FB = faba beans; FB-SPI = faba beans and *S. platensis* microalgae.

[2] No significant differences in DMI in Exp. 1-3. In Exp. 4, the effect of partial substitution of rapeseed meal and faba beans with *S. platensis* microalgae (RSM + FB vs. RSM-SPI + FB-SPI): $P<0.05$.

[3] Crude fat concentration of total mixed ratio.

up to 450 g/kg DM. Also Hintz et al. (1966) noticed feed intake problems on wethers when feeding microalgae with crude fat concentration of 250 g/kg DM. Costa et al. (2016) reported that on *Bos indicus* steer diets based on poor quality forage, *S. platensis* resulted in similar DMI compared to cottonseed meal, whereas *C. pyrenoidosa* and *Dunaliella salina* resulted in approximately 0.62 and 1.5 kg/d, respectively, lower DMI than cottonseed meal. Feed refusal was observed on *D. salina,* on which steers were willing to consume only 17.5% of the planned microalgae dosage.

The poorer intake of microalgae can be caused by taste or odour properties, nutritive characteristics or physical structure of dry powdery algae, or any combination of these. Van Emon et al. (2015) pointed out that the lower palatability of diets containing large amounts of microalgae might be caused by the dry nature of algae diets (DM >900 g/kg) and this could have been overcome by increasing the moisture content. However, this was unlikely a

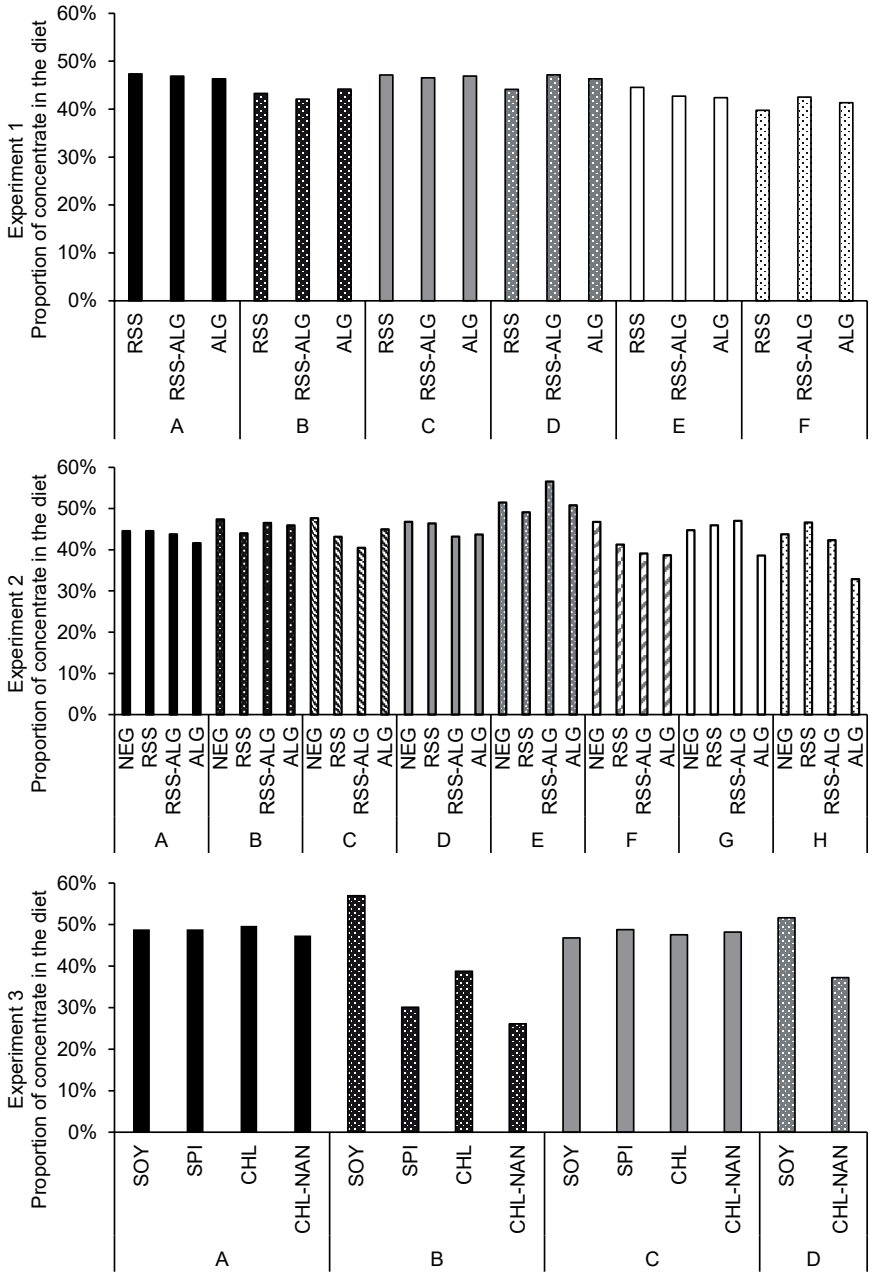


Figure 5 The proportion of concentrate in the diets in Experiments 1 to 3 (I-II) on each animal (A-H). Treatments in Exp. 1: RSS = rapeseed supplement; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* microalgae; RSS-ALG = mixture of RSS and ALG. Treatments in Exp. 2: NEG = no protein feed; RSS = rapeseed supplement; ALG = *S. platensis* microalgae; RSS-ALG = mixture of RSS and ALG. Treatments in Exp. 3: SPI = *S. platensis* microalgae; CHL = *C. vulgaris* microalgae; CHL-NAN = mixture of *C. vulgaris* and *Nannochloropsis gaditana* microalgae. In Exp. 3, cow D was removed from the experiment after 2nd period due to feed intake problems.

cause of poor palatability of microalgae diets in current experiments as a small amount of water was added to concentrates containing microalgae in all experiments in which concentrates and silage were fed separately (Exp. 1 to 3; I-II) to bind powdery algae on pellets. The resulting average DM content of concentrates was 755 to 786 g/kg in Experiments 1 to 3. To some extent, though, this caused the breakdown of the pelleted structure of concentrates, which in turn might have affected voluntary concentrate intake. However, this does not explain the differences in feed intake response between Experiment 1 and Experiments 2 to 4.

High dietary fat concentration has a potential to decrease DMI (Onetti and Grummer, 2004), but this unlikely explains the poor intake of microalgae in Experiments 2 to 4 for the following reasons. First, the crude fat concentration of concentrates in Experiments 1 to 3 was moderate even on microalgae diets, being 39.2 to 60.0 g/kg DM (Table 6). According to Huhtanen et al. (2008b), the concentrate crude fat concentration of 43 to 57 g/kg DM has quantitatively minimal effects on silage DM intake. They reported decreases of 0.025 kg in DMI per 1 g/kg DM increase in dietary crude fat concentration. Second, when concentrates high in fat and forage were given separately, the intake of forage but not concentrate supplement has been decreased (Moate et al., 2013; Kairenius et al., 2015). The mechanism behind this can relate to e.g. negative effects of dietary fat on ruminal fermentation (Allen, 2000).

Other concentrate related factors known to affect DMI of ruminants include the fermentability of OM and the ratios of VFA produced, and dietary mineral concentration (Allen, 2000). The effect of the first two will be discussed in sections 4.2.2 and 4.4. Of minerals, especially electrolytes sodium (Na) and potassium (K) are able to increase the osmolality of rumen fluid, which can decrease feed intake if too high (Allen, 2000). In sheep, single pulse dose of 2.37 to 50.0 g NaCl has linearly decreased DMI by 5.34 g/g NaCl after 30 min of dosing (Carter and Grovum, 1990). The K concentration of microalgae [12.3, 5.40 and 12.4 g K/kg DM for *S. platensis* (Exp. 1 to 4), *C. vulgaris* (Exp. 1 and 3) and *N. gaditana* (Exp. 3), respectively] was similar or even lower than for rapeseed meal and faba beans (12.9 and 14.1 g K/kg DM, respectively) in Exp. 4 (Lamminen et al., unpublished). In this analysis, the determination of Na was not possible due to low recovery of Na in microwave digestion. Nevertheless, in literature Na concentrations of *S. platensis* (1.2 to 18 g Na/kg DM; Ortega-Calvo et al., 1993; Costa et al., 2016), *C. vulgaris* (0.3 to 13 g Na/kg DM; Ortega-Calvo et al., 1993; Dineshkumar et al., 2017), *Nannochloropsis* sp. (194 g Na/kg DM; Costa et al., 2016), *N. oculata* (42 g Na/kg DM; Costa et al., 2016) and *N. oceanica* (2.9 g/kg DM; He et al., 2017) have deviated largely from those of rapeseed meal, soya bean meal and faba beans (0.0 to 0.8 g Na/kg DM; Heuzé et al., 2017, 2018a, 2018b). The Na, K and Cl in *N. oceanica* has been reported to be virtually completely water-soluble (He et al., 2017), increasing further their potential to influence the osmolality of rumen fluid. Assuming Na concentrations of protein feeds similar to literature as stated above, the theoretical Na intake from protein

feeds in Exp. 3 could have been 1.34 to 20.2 g Na/d for *S. platensis*, 0.41 to 17.6 g Na/d for *C. vulgaris*, 2.60 to 168 g Na/d for mixture of *C. vulgaris* and *N. gaditana* and 0 to 1.48 g Na/d for soya bean supplement. Thus, this might be one explanation for lower intake of microalgae than conventional protein feeds, and mixture of *C. vulgaris* and *N. gaditana* than other microalgae. However, while the ash concentration of *N. gaditana* in Exp. 3 was higher than other protein feeds, it was similar or even lower for *S. platensis* and *C. vulgaris* than for soya bean supplement. It also remains unclear whether constantly higher dietary mineral concentration with unlimited access to drinking water has similar effect on DMI as sudden pulse doses administrated directly to reticulorumen.

Of the algae used in current experiments, *N. gaditana* had a clear fishy odour for human perception, whereas the odour of *S. platensis* and *C. vulgaris* was more neutral. This is relevant because feed selection of ruminants is affected by odour and taste properties of the feed (Cannas et al., 2009). Ruminants are also relatively conservative eaters that prefer familiarity over novelty (Rapisarda et al., 2012). When comparing different plant based concentrates, Rapisarda et al. (2012) noticed that sheep preferred feeds rich in aldehydes (green, fruity and rancid aromas), and avoided feeds rich in sulphuric (garlic, meaty and fishy aromas) and terpenic (solvent, spice and wood aromas) compounds. Van Durme et al. (2013) noticed no fishy odour nor sulphuric aroma compounds in *N. oculata* paste and the concentration of terpenes was low, instead, this microalgae was dominated by 'grassy, vegetable, cucumber' flavours. However, processing and storage conditions of microalgae can affect their odour and taste properties. For example, microbial degradation of sulphuric-AA results in sulphuric aroma compounds (Seefeldt and Weimer, 2000) and several different aroma compounds can be produced when unsaturated FA are being oxidised (Sérot et al., 2002). The taste, odour and novelty factors may to some extent explain the DMI responses to microalgae in Experiments 2 to 4, but yet again, the reason for difference in Experiment 1 remains unclear.

As was evident in Experiment 4 (III), TMR feeding did not solve the feed intake problems related to feed use of microalgae, despite of the fact that it has been reported to decrease sorting behaviour of animals in comparison to separate feeding of concentrates and forages, and has subsequently lead to more balanced nutrient intake (DeVries and von Keyserlingk, 2009; Greter et al., 2010). Hintz et al. (1966) were able to improve the palatability of microalgae by pelleting it as a part of the whole hay-based dietary ration. Deodorisation with ethanol has been shown to be a promising method to decrease the odour and increase sensorial acceptability of microalgae for human consumption with minimal impact on nutritive characteristics of microalgae (Cuellar-Bermúdez et al., 2017). Improving the palatability of microalgae can have great benefits not only considering animal welfare, but also productivity, especially if concentrates and forage are fed separately like in Experiments 1 to 3 (I-II).

4.2.2 DIGESTIBILITY OF NUTRIENTS

Protein supplementation

The apparent total tract digestibility of DM, organic matter (OM), NDF and CP estimated using AIA as a marker was increased in Experiment 2 (I) by protein supplementation. The same was observed in Korhonen et al. (2002) with fishmeal, soya bean meal and corn gluten meal as protein sources. The apparent digestibility of CP typically increases with increasing CP intake (Spanghero and Kowalski, 1997) because metabolic faecal N excretion rate per kg of DMI stays relatively constant (Waldo and Glenn, 1984) and does not increase with increasing CP intake as much as N of feed origin in feed and faeces. The improvement in apparent digestibility of OM and NDF in Experiment 2 (I) was much larger in magnitude than was estimated in Huhtanen et al. (2011) for rapeseed meal, soya bean meal, or mixture of soya bean and fish meal. The improvements in digestibility of OM and NDF by protein supplementation were on average 0.58 and 1.8 g/kg per 1 g/kg DM increase of diet CP concentration in I (Exp. 2), respectively, whereas Huhtanen et al. (2011) reported the respective increases of 0.31 and 0.64 for rapeseed meal. This difference was likely caused by the lower digestible OM content of silage and concentration of CP in unsupplemented diet in Experiment 2 (I) than on average in the diets used in the meta-analysis of Huhtanen et al. (2011).

In the light of positive effect of protein supplementation on nutrient digestibility, the tendency for increased silage intake with protein supplementation in Experiment 2 (I) is logical, especially when also the intake of EAA, total AA and ME was increased. However, the expected effect of protein supplementation on total DMI was not realised due to the negative effect of microalgae on concentrate intake. Oldham (1984) suggested that the positive effect of protein supplementation on DMI is related to faster rate of fibre digestion in the rumen that is stimulated by increased N, but especially AA supply. In turn, this would decrease the distension in gastrointestinal tract and allow larger amounts of DM to be consumed. On the other hand, improved AA supply *per se* seems not to affect DMI (Allen, 2000). In contrast, this response is rather related to the balance of AA and energy. Accordingly, Huhtanen et al. (2011) suggested that the improved AA to ME ratio at tissue level could contribute to increased milk production and subsequently pull feed intake.

Microalgae vs. conventional protein feeds

In most cases the substitution of conventional protein feeds with microalgae resulted in similar apparent total tract digestibility of nutrients as conventional protein feeds. Thus, the poor palatability of microalgae and the differences in DMI between Experiment 1 (I) and Experiments 2 to 4 (I-III) are unlikely explained by digestibility of nutrients. The only digestibility responses noticed were in Experiment 1 (I) with a tendency for quadratic response of OM digestibility to microalgae inclusion in the diet. In that case

the 50 % substitution rate of microalgae for rapeseed meal resulted in lower OM digestibility than pure rapeseed meal and microalgae. Statistically significant but biologically negligible effect was seen also on digestibility of starch in Experiment 4 (III), with increasing digestibility on rapeseed meal, but decreasing on faba beans by microalgae inclusion in the diet. Han and McCormic (2014) reported that the defatted microalgae residues of *Chlorella* sp. and many other microalgae resulted in lower *in vitro* gas production than soya bean meal and alfalfa hay, indicating less fermentable carbohydrate composition of microalgae. Moreover, the fast fermenting fraction of *Chlorella* sp. was similar to that of soya bean meal, whereas the slow fermenting fraction in *Chlorella* sp. was 35 % lower than that of soya bean meal. On the other hand, Lodge-Ivey et al. (2013) observed that the *in vitro* OM digestibility of *Chlorella* spp. and *N. salina* varied being 11.7 %-units higher to 9.1 %-units lower than that of soya bean meal depending on the cultivation and harvesting method of microalgae. However, they did not compare the digestibility of these two microalgae. Drewery et al. (2014) reported that defatted *Chlorella* sp. residue resulted in similar ruminal digestibility of NDF, and apparent total tract digestibility of OM and NDF than cottonseed meal in steers consuming oat straw. In sheep, the substitution of ground soyhulls or corn with algae meal consisting of soyhulls and partially defatted *Prototheca moriformis* microalgae resulted in linear decreases in the digestibility of DM and OM (Stokes et al., 2015). The digestibility of NDF and ADF were also decreased when algae meal substituted for ground soyhulls, but were increased when algae meal substituted for corn (Stokes et al., 2015).

Different microalgae species were hypothesised to induce differences in nutrient digestibility due to differences in their cell wall rigidity. However, no signs of this were observed in Experiment 3 (II). On the other hand, it may well be that the measurement of apparent total tract digestibility is not sensitive enough to pinpoint differences in digestibility of individual feeds, especially when the dietary concentrate:forage ratio is simultaneously affected and daily microalgae amount was relatively low in comparison to other dietary components. It has been demonstrated in biogas production, which mimics ruminal fermentation of feedstuffs via microbial digestion, that the digestibility of many cyanobacteria species is higher than that of *C. vulgaris* (Mendez et al., 2015), and the anaerobic digestibility of *Chlorella* sp. and *Nannochloropsis* sp. can be hindered by their rigid cell wall (Bohutskyi et al., 2014). Although the results obtained in biogas production cannot be applied directly to fermentation in ruminant animal, there is some support in animal nutrition trials to differences in nutrient digestibility of different microalgae species. Costa et al. (2016) reported comparable apparent total tract digestibility of DM in cottonseed meal, *C. pyrenoidosa* and *S. platensis*, whereas that of *Dunaliella salina* was lower than in the first two. Han and McCormick (2014) observed that the *in vitro* fast fermenting fraction of *Chlorella* sp. was higher than that of *Selenastrum capricornutum* and *Scenedesmus dimorphus*, whereas that of *Scenedesmus* sp. and *Thalassiosira*

weissflogii were intermediate. On the other hand, the *in vitro* slow fermenting fraction was intermediate in *Chlorella* sp.

4.3 MILK PRODUCTION

4.3.1 MILK YIELD

Protein supplementation

Milk yield was unaffected by protein supplementation in Experiment 2 (I), which is logical given the unaffected DMI. DMI is typically the main factor affecting milk yield of dairy cows followed by the intake of metabolisable protein and energy components such as fat and starch (Hristov et al., 2004). Depending on the predictive model, the intakes of DM (Hristov et al., 2004), CP, metabolisable protein (Hristov et al., 2004; Huhtanen and Hristov, 2009), soluble protein (Hristov et al., 2004) and total digestible nutrients (Huhtanen and Hristov, 2009) have been suggested to be the main factors determining milk protein yield. In Experiment 2 (I), protein supplementation increased numerically milk, ECM and milk protein yield on average 0.83 kg/d, 0.97 kg/d and 15.7 g/d, respectively. In Korhonen et al. (2002) milk, lactose and protein yields were increased by protein supplementation when DMI was limited to 95 % of *ad libitum* intake and DMI did not differ between unsupplemented and protein supplemented diets. In that case, the increased digestibility of nutrients, AA supply or improved AA composition seemed to explain the higher milk production level on protein supplemented diets. They did not notice any effects on ECM.

The milk (2.3 kg), ECM (2.8 kg) and milk protein (82 g) responses per 1 kg increase in CP intake obtained in Experiment 2 (I) with rapeseed meal were lower than the corresponding values of 3.4 kg, 3.7 kg and 136 g on rapeseed meal supplemented diets in Huhtanen et al. (2011). However, the results were in agreement with Puhakka et al. (2016) and Ramin et al. (2017). Huhtanen (1998) noted that the milk protein responses to the supplementary rapeseed meal were not related to dietary CP concentration, thus it is likely not the cause for the lower production responses than in the meta-analysis of Huhtanen et al. (2011). The cows in Experiment 2 were relatively late in lactation (190 days in milk), but it is unsure how this affects milk production responses. Kalscheur et al. (1999) noticed that the milk, protein and fat yields of dairy cows were increased in early lactation with increasing dietary CP concentration, but not in mid- or late lactation. On the other hand, Saarisalo et al. (2002) reported that rapeseed meal supplementation in late lactation resulted in an equal milk production response (1.0 kg per 1 kg DM of rapeseed meal) as in early or mid-lactation (1.05 kg per 1 kg DM of rapeseed meal; Huhtanen, 1998).

Microalgae vs. conventional protein feeds

There were relatively large individual variation in milk production responses to substitution of conventional protein feeds with microalgae, which was caused by inconsistencies in feed intake (Figure 5). The partial or complete substitution of rapeseed meal with *S. platensis* (Exp. 2 and 4; I, III) or mixture of *S. platensis* and *C. vulgaris* (Exp. 1; I) did not affect statistically milk or ECM yield (Figure 6) in Experiments 1 and 2 (I). However, in Experiment 4 (III) the partial substitution of rapeseed meal or faba beans with *S. platensis* decreased milk yield on rapeseed meal, but increased on faba bean supplemented diets. In Experiment 2 (I), the responses of milk, ECM and protein per 1 kg increase in dietary CP intake were especially low in the diet with 50 % substitution rate of rapeseed meal by *S. platensis*, being 0.96 kg, 0.72 kg and -2.41 g for milk, ECM and milk protein yield, respectively. Interestingly, in Experiment 1 (I) the same 50 % substitution rate of rapeseed meal by the mixture of *S. platensis* and *C. vulgaris* tended to result in higher milk yield than diets with pure rapeseed meal or mixture of *S. platensis* and *C. vulgaris*.

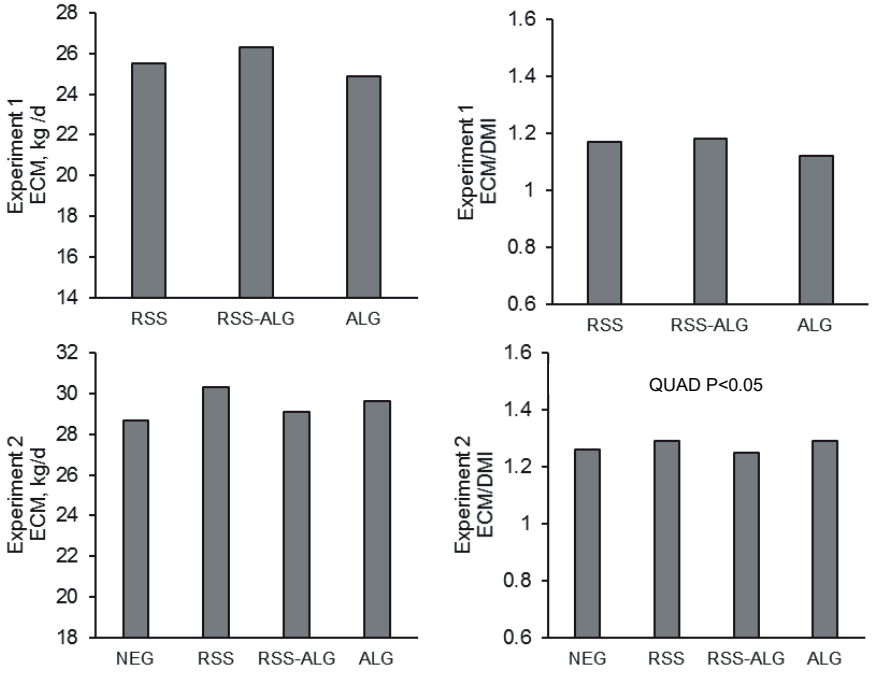


Figure 6 Energy corrected milk yield (ECM) and ratio of energy corrected milk and dry matter intake (ECM/DMI) in Experiments 1 and 2 (I). Treatments in Exp. 1 and 2: NEG = no protein feed; RSS = rapeseed supplement; ALG = *Spirulina platensis* (Exp. 2) or mixture of *S. platensis* and *C. vulgaris* (Exp. 1); RSS-ALG = mixture of RSS and ALG. Orthogonal contrasts in Exp. 2: QUAD = quadratic response to substitution of rapeseed with *S. platensis*. Standard error of mean (SEM) for ECM: Exp. 1 (1.39), Exp. 2 (1.39). SEM for ECM/DMI: Exp. 1 (0.074), Exp. 2 (0.052).

The complete substitution of soya bean meal with *S. platensis*, *C. vulgaris* or mixture of *C. vulgaris* and *N. gaditana* did not affect statistically milk or ECM yields (Figure 7, Exp. 3; II). However, the numerical differences were relatively large, microalgae diets resulting in on average 1.23 kg/d, 2.17 kg/d and 37.7 g/d higher milk, ECM and protein yields, respectively, than soya bean. Numerically the highest milk yields were recorded on *S. platensis*.

The increased milk production and ECM:DMI ratio when *S. platensis* substituted for half of the protein in faba beans indicates the complementary nutritive value of *S. platensis* and faba beans. This cannot be explained by changes in total tract apparent digestibility of nutrients. Moreover, in general, it remains unclear to which extent the milk production response of microalgae was affected by their poorer palatability in relation to conventional protein feeds. The relative difference in milk yield responses to microalgae inclusion between rapeseed and grain legume diets is logical given the higher milk production response of rapeseed meal than that of soya bean meal (Huhtanen

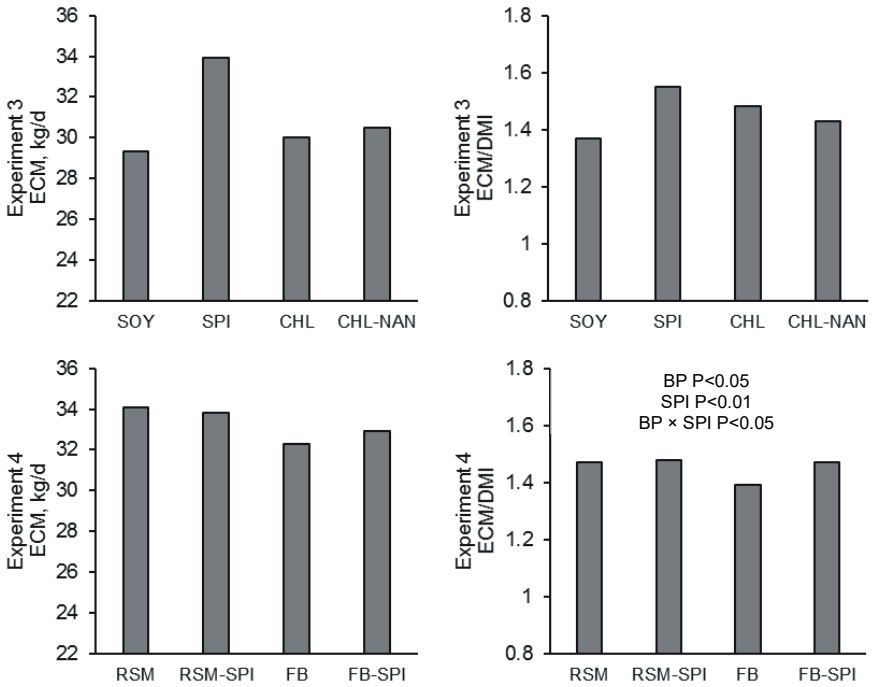


Figure 7 Energy corrected milk yield (ECM) and ratio of energy corrected milk and dry matter intake (ECM/DMI) in Experiments 3 and 4 (II, III). Treatments in Exp. 3: SOY = soya bean meal; SPI = *Spirulina platensis*; CHL = *Chlorella vulgaris*; CHL-NAN = mixture of *C. vulgaris* and *Nannochloropsis gaditana*. Exp. 4: RSM = rapeseed meal; RSM-SPI = rapeseed meal and *S. platensis*; FB = faba beans; FB-SPI = faba beans and *S. platensis*. Orthogonal contrasts in Exp. 4: BP = RSM + RSM-SPI vs. FB + FB-SPI; SPI = RSM + FB vs. RSM-SPI + FB-SPI; BP ×SPI = interaction between BP and SPI. Standard error of mean (SEM) for ECM: Exp. 3 (2.02), Exp. 4 (0.86). ECM/DMI: Exp. 3 (0.130), Exp. 4 (0.044). For treatments SOY and CHL-NAN in Experiment 3 SEM must be multiplied by 0.8306 due to missing observations on other treatments.

et al., 2011) and that of faba beans (Puhakka et al., 2016), which might be at least partially be caused by higher DMI. Moreover, the increased milk yield in response to microalgae inclusion to grain legume diets implied also potential to improve milk production response of these feeds with supplementation of methionine-rich feeds, such as microalgae. Previously, methionine limitation has been suggested as a reason for lower milk production response of faba beans than that of rapeseed meal (Puhakka et al., 2016). On soya bean supplemented diets, methionine supplementation has increased fat corrected milk yield (Schingoethe et al., 1988; Broderick et al., 2009), and milk fat (Broderick et al., 2009) and protein yields (Schingoethe et al., 1988; Pisulewski et al., 1996; Armentano et al., 1997; Broderick et al., 2009).

4.3.2 MILK COMPOSITION

Protein supplementation

Protein supplementation did not affect milk composition in Experiment 2 (I). Previously, protein supplementation has resulted in increases in milk protein concentration (Korhonen et al., 2002), and decreases (Choi et al., 2002; Korhonen et al., 2002) or increases (M'Hamed et al., 2001) in milk fat concentration. Korhonen et al. (2002) explained the decrease of milk fat concentration with the dilution effect, i.e. synthesised milk fat was secreted into a larger volume of milk. Indeed, milk protein and fat concentration are typically negatively correlated with milk yield (Oldham and Sutton, 1980). Especially the lack of response on milk protein concentration in Experiment 2 (I) was unexpected given the increased EAA and total AA intakes with protein supplementation. In general, milk protein concentration is affected by the overall quantity of absorbable AA in intestines and availability at the mammary gland, as well as the mammary supply of EAA and AA limiting milk protein production (Murphy and O'Mara, 1993). However, the relatively low digestible OM concentration of the silage and possible suboptimal supply of energy in Experiment 2 (I) might have limited the utilisation of supplementary protein in milk production.

Microalgae vs. conventional protein feeds

The most notable effects of microalgae supplementation to milk composition relate to changes in milk fat concentration especially on *S. platensis*. In Experiment 4 (III), milk protein and fat concentrations were increased by 0.6 and 2.0 g/kg, respectively, when rapeseed meal was partially substituted with *S. platensis*. The opposite was true for faba bean supplemented diet, with decreases of 0.7 and 1.1 g/kg in milk protein and fat concentrations, respectively. Due to these changes in milk fat concentration, ECM yield remained unaffected in Experiment 4 when rapeseed meal and faba beans were partially substituted with *S. platensis* even though milk yield was decreased on rapeseed meal, and increased on faba bean supplemented diets. In Exp. 3 (II), microalgae diets tended to result in higher milk fat

concentration than soya bean meal, mainly due to the marked increase on *S. platensis*. The milk fat concentration was also higher and milk fat yield tended to be higher on *S. platensis* than on diets consisting of *C. vulgaris,* and mixture of *C. vulgaris* and *N. gaditana*. On *S. platensis,* milk fat concentration and yield were 3.47 g/kg and 230 g/d higher than on other diets. On the other hand, milk fat concentration was unaffected when rapeseed meal was substituted with *S. platensis* (Exp. 2; I) or mixture of *S. platensis* and *C. vulgaris* (Exp. 1; I).

Milk fat is synthesised from plasma acetic acid, β-hydroxybutyrate (BHBA), non-esterified FA (NEFA) and preformed FA originating from diet and metabolised from tissue reserves (Chilliard et al., 2000). Of these, acetic acid and BHBA can originate from acetate or butyrate intensive ruminal fermentation, and NEFA and BHBA from body lipid mobilisation (Chilliard et al., 2000). The precursors of milk fat can explain the reasons for changes in milk fat concentration in Experiments 3 and 4 (II, III). In Experiment 3, arterial acetic acid and NEFA concentrations tended to increase when soya bean meal was substituted with microalgae. This also increased the mammary uptake of acetic acid but did not affect that of BHBA or NEFA. Increased proportion of silage on microalgae diets may have favoured the growth of acetate producing bacteria (France and Dijkstra, 2005). On the other hand, energy balance also tended to decrease and reached more negative values in Experiment 3 (II) on microalgae diets compared to soya bean meal. Negative energy balance is known to affect milk fat to protein ratio by increasing milk fat concentration and decreasing that of milk protein (Duffield et al., 1997).

In Experiment 4 (III), the changes in milk fat concentration in response to *S. platensis* inclusion in the diet were not related to ruminal supply of acetate and butyrate. The proportion of actate and ratio of acetate and butyrate to propionate was decreased by *S. platensis* inclusion on both rapeseed and faba bean supplemented diets, but the response of milk fat concentration to *S. platensis* inclusion differed between these two. Moreover, no changes were observed in arterial acetic acid, NEFA or BHBA. In Experiment 4 (III), milk fat may, however, reflect the concentration/dilution effect of milk solids due to changes in milk yield in response to *S. platensis* inclusion in the the diet.

The reason for increased milk fat production may also be related to protein characteristics of *S. platensis*, as milk fat concentration typically increases with post-ruminal infusions of methionine (Varvikko et al., 1999; Zanton et al., 2014), but also with dietary methionine supplementation (Zanton et al., 2014). Varvikko et al. (1999) also reported increasing ECM yields with increasing abomasal supply of infused methionine. Moreover, Choi et al. (2002) noticed that milk fat concentration was higher for rapidly degradable protein diets than for slowly degradable protein diets. However, in Mutsvangwa et al. (2016) the change in ruminal protein degradability of rapeseed meal had no effect on 3.5% fat corrected milk yield or milk protein or fat concentration. In addition, Haque et al. (2012) observed decreasing milk

fat concentrations when duodenal supply of AA was balanced. Simultaneously, milk and protein yield and milk protein concentration were increased.

4.4 ENERGY METABOLISM

In this thesis, the energy metabolism of lactating dairy cows was evaluated on the basis of ruminal fermentation (Exp. 2 and 4; I, III) and the plasma concentrations of energy metabolites (Exp. 1 to 4; I-III). In addition, the sufficiency of energy supply was estimated in all experiments on the basis of ME intake and requirements for milk production. In general, rumen fermentation pattern observed in Experiment 2 (I) and 4 (III) was characterised with low molar proportion of propionate and high proportion of lipogenic volatile FA (VFA) acetate and butyrate. This is typical for diets based on restrictively fermented grass silage (Huhtanen, 1998).

Protein supplementation

The major ruminal fermentation pattern was not affected by protein supplementation in Experiment 2 (I). This is logical since the manipulation of ruminal fermentation pattern is generally considered difficult on restrictively fermented grass silage-based diets (Huhtanen, 1998). The lack of major VFA responses to protein supplementation are also in agreement with Korhonen et al. (2002). In Experiment 2 (I), there were, however, slight increases in the molar proportions of minor VFA isobutyrate, isovalerate and caproate. In general, the average arterial glucose and NEFA concentrations were in normal levels for lactating dairy cows (Cozzi et al., 2011) in all experiments. This is also in agreement with the positive calculated ME balance in Experiment 2 (I).

Microalgae vs. conventional protein feeds

In general, the responses of ruminal VFA to *S. platensis* inclusion in the diet in Experiments 2 (I) and 4 (III) were variable but relatively small. These responses depended at least partially on the source of protein in the basal diet (rapeseed meal or faba beans). In addition, the results might also be confounded by the extent and pattern of feed intake depression induced by *S. platensis* inclusion in the diet. The ruminal concentration of VFA or pH did not explain the difference in DMI between conventional protein feeds and *S. platensis*. Ruminal pH was slightly decreased and total VFA concentration slightly increased in Experiment 4 with *S. platensis* inclusion in the diet. However, all values in Experiment 2 and 4 were in normal physiological range and indicated no signs of subacute ruminal acidosis (Morgante et al., 2007; Plaizier et al., 2008). Subacute ruminal acidosis can decrease DMI via reduced fiber digestibility, increased VFA production and especially propionate, or increased rumen osmolality (Allen, 2000).

In Experiment 2 (I), the substitution of rapeseed meal with *S. platensis* had no effect on molar proportions of major VFA in the rumen. On the other hand,

in Experiment 4 (III), *S. platensis* inclusion in the diet increased ruminal proportion of propionate on both rapeseed meal and faba bean supplemented diets. The molar proportion of acetate was decreased on rapeseed meal, but not on faba bean supplemented diets. Also the ratios of acetate to propionate, and acetate and butyrate to propionate were decreased when rapeseed meal and faba beans were partially substituted with *S. platensis*. Ruminal fermentation was not studied in Experiment 3 (II), however, in this experiment arterial concentrations of acetic acid were increased especially on *S. platensis* when soya bean meal was substituted with microalgae. This can originate from more acetate intensive ruminal fermentation, because increased ruminal supply of acetate has been shown to increase arterial acetic acid concentrations (Huntington et al., 1983).

The reason for small but still significant increase on ruminal proportion of propionate on *S. platensis* diet in Experiment 4 (III) remains unclear. Increased ruminal proportion of propionate is usually associated with increased proportion of concentrate in the diet (e.g. Van Soest, 1994; Sutton et al., 2003; Agle et al., 2010). This does not explain the responses in Experiment 4 due to fixed concentrate:forage ratio in TMR feeding. On forage-based diets, propionate is mainly produced via succinate pathway by cellulolytic bacteria *Fibrobacter succinogenes*, whereas on diets high in soluble carbohydrates, acrylate pathway is favoured in conversion of starch to lactate and to propionate (Van Soest, 1994; Moss et al., 2000). Inclusion of *S. platensis* in the diet increased starch intake (0.2 kg/d) on rapeseed meal supplemented diets. However, when faba beans were partially substituted with *S. platensis*, the increased ruminal propionate proportion was observed even when the starch intake was decreased (-0.2 kg/d). As propionate formation functions as a hydrogen sink in the rumen, the increased ruminal production of propionate has potential to decrease methane emissions (Van Soest, 1994; Moss et al., 2000). However, the observed change in ruminal proportion of propionate in Experiment 4 (III) by *S. platensis* inclusion in the diet was so minimal that it likely has very limited significance on mitigating methane emissions.

Although other VFA than acetate, butyrate and propionate have minor importance for energy supply of ruminant animals, the small but consistent effects of *S. platensis* inclusion on rapeseed meal supplemented diets on ruminal branched-chain VFA are notable. Both in Experiments 2 (I) and 4 (III), molar proportions of isobutyrate and isovalerate were increased in rumen when rapeseed meal was substituted with *S. platensis*. Isobutyrate and isovalerate origin from dietary supply of valine and leucine, respectively, (Van Soest, 1994), the intake of which was increased in Exp. 2 and 4 by *S. platensis* inclusion on the diet.

The substitution of conventional protein feeds with microalgae had no consistent effect on arterial concentrations of energy metabolites. Moreover, these responses depended mainly on the effect of microalgae on feed intake but not ME balance. Microalgae inclusion in the diet had no effects or only minor effects on arterial concentrations of acetic acid, BHBA, glucose, insulin

and NEFA in Experiments 1 (I) and 4 (III). In Experiment 1, the quantity and composition of DMI was unaffected by the dietary treatments. In Experiment 4, the composition of DMI was unaffected due to TMR feeding, but total DMI was decreased (0.65 kg/d) by microalgae inclusion in the diet. In Experiments 2 (I) and 3 (II) with unchanged total DMI but decreased concentrate:forage ratio, microalgae inclusion in the diet increased or tended to increase arterial NEFA concentration. In addition, arterial BHBA tended to increase in Experiment 2 (I), and it was numerically twofold higher on *S. platensis* than on other treatments in Experiment 3 (II). Animals were estimated to be on positive ME balance on all treatments in Experiments 1 and 2 (I), despite of feed intake problems on microalgae diets in Experiment 2. In contrast, negative or close to zero ME balance were estimated on all microalgae treatments in Experiment 3 (II), and on diet constituting of mixture of rapeseed meal and *S. platensis* in Experiment 4 (III).

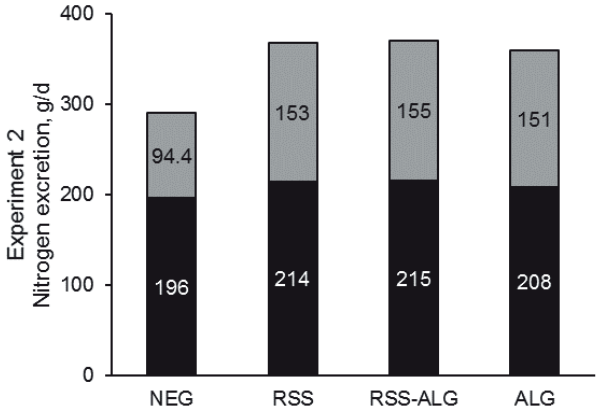
4.5 NITROGEN AND AMINO ACID METABOLISM

4.5.1 INTAKE AND UTILISATION OF NITROGEN

In this thesis, the sufficiency of N supply and utilisation of dietary N was evaluated on the basis of N intake, ruminal NH_4-N concentration (only in Exp. 2 and 4; I, III), the utilisation of N (NUE, total N excretion to environment and human-edible protein efficiency), milk protein yield, urea concentration in milk and urine, and calculated N balance. Urinary and faecal excretion of N to environment were measured in Experiments 2 to 4 (I-III).

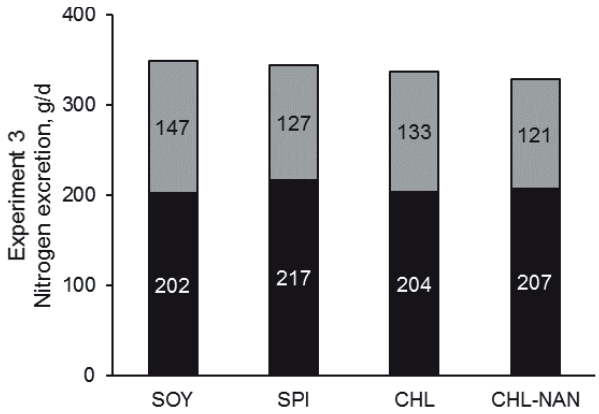
Protein supplementation

Protein supplementation (Exp. 2; I) decreased NUE, increased ruminal NH_4-N concentration, milk urea N (MUN) and secretion of N to environment (urinary, faecal and total N; Figure 8). This is a common response to increasing dietary CP concentration and the same has been observed e.g. in Castillo et al. (2000), and Huhtanen and Hristov (2009). Indeed, dietary CP concentration is the main determinant affecting NUE (Huhtanen et al., 2008a; Spek et al., 2013). However, NUE is not related to N intake *per se* (Dijkstra et al., 2013c), rather it depends on the adequate dietary balance of nitrogen and energy in relation to animal requirements (Oldham, 1984). In Experiment 2 (I), the CP concentration of the unsupplemented diet was 125 g/kg DM, whereas on protein supplemented diets it was on average 152 g/kg DM in Experiment 1 (I), 149 g/kg DM in Experiment 2 (I), 153 g/kg DM in Experiment 3 (II), and 168 g/kg DM in Experiment 4 (III). The NUE was in the unsupplemented diet (Exp. 2; I) 0.343, whereas it was on protein supplemented diets on average 0.250 in Experiment 1 (I), 0.289 in Experiment 2 (I), 0.294 in Experiment 3 (II), and 0.280 in Experiment 4 (III). These values are at the upper end of NUE values reported in literature. In North or Northwest European datasets the



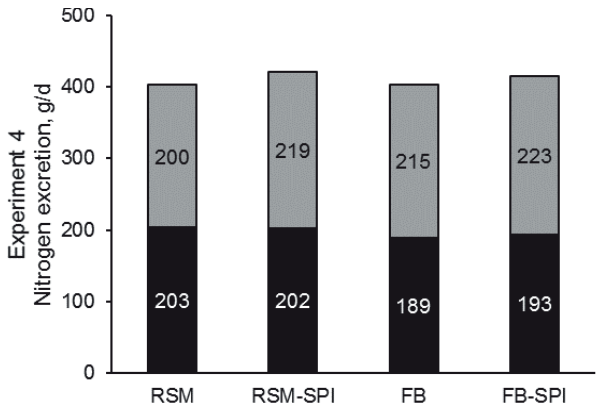
Statistical significance	
Urinary N	PROTEIN: P<0.001
Faecal N	PROTEIN: P<0.05
Total N	PROTEIN: P<0.001

SEM for urinary N: 6.0; for faecal N: 6.2 and for total N: 13.5.



Statistical significance	
Urinary N	SOY vs. algae: P<0.05
Faecal N	
Total N	

SEM for urinary N: 12.1; for faecal N: 18.0 and for total N: 28.4. SEM for treatments SOY and CHL-NAN must be multiplied by 0.8306.



Statistical significance	
Urinary N	BP: P<0.05 SPI: P<0.01
Faecal N	BP: P<0.05
Total N	SPI: P<0.05

SEM for urinary N: 7.4; for faecal N: 6.7 and for total N: 12.7.

Figure 8 Nitrogen excretion in urine and faeces (g N/d) in Experiments 2 to 4 (I-III).Treatments in Exp. 2: NEG = no protein feed; RSS = rapeseed supplement; ALG = *Spirulina platensis*; RSS-ALG = mixture of RSS and ALG. Exp. 3: SOY = soya bean meal; SPI = *S. platensis*; CHL = *C. vulgaris*; CHL-NAN = mixture of *C. vulgaris* and *Nannochloropsis gaditana*. Exp. 4: RSM = rapeseed meal; RSM-SPI = rapeseed meal and *S. platensis*; FB = faba beans; FB-SPI = faba beans and *S. platensis*. Orthogonal contrasts in Exp. 2: PROTEIN = NEG vs. RSS + RSS-ALG + ALG ; LIN = linear responses to substitution of rapeseed with *S. platensis*. Exp. 3: SOY vs. Algae = SOY vs. SPI + CHL + CHL-NAN. Exp. 4: BP = RSM + RSM-SPI vs. FB + FB-SPI; SPI = RSM + FB vs. RSM-SPI + FB-SPI.

average NUE in diets has been 0.274 to 0.277, and North American datasets 0.247 to 0.261 (Huhtanen and Hristov, 2009; Spek et al., 2013). Calsamiglia et al. (2010) reported the highest and lowest quartile of NUE to be 0.210 and 0.320 in European, and 0.220 and 0.328 in American dairy cow diets.

However, there is much room for improvement in NUE in general, as Dijkstra et al. (2013c) estimated that the theoretical maximum in NUE would be 0.43 in dairy cows. This estimation was based on assumption of minimum inevitable N losses of 89 and 174 g/d in faeces and urine, respectively, for a cow with DMI 24.1 kg/d and producing 40 kg/d of fat and protein corrected milk. In Experiments 2 to 4 (I-III), the faecal and urinary N losses varied from 189 to 217 g N/d and 94.4 to 223 g N/d, respectively. The lower urinary N losses in current experiments than the minimum suggested by Dijkstra et al. (2013c) is likely explained by potential inaccuracies in the estimation of urinary volume from spot sampling. In that case, also the accuracy of estimation of N balance (= N intake – N in milk – N in faeces – N in urine) would have been affected. However, this still allows comparison of treatment effects on N excretion within experiments. In addition, the average urine N concentrations (4.99 to 7.00 g N/l) on dietary treatments in Experiments 2 to 4 (I-III) was in agreement with the concentrations summarised by Dijkstra et al. (2013b).

Despite of clear environmental benefits of not supplementing dairy cow diets with protein feeds, animal health and welfare costs may be related to protein unsupplemented diets. The cows on unsupplemented diet (Exp. 2; I) likely suffered from shortage of N for ruminal microbes as indicated by low MUN (6.33 mg/dl) and ruminal NH_4-N concentration (2.52 mmol/l). Broderick et al. (2010) estimated that zero rumen N balance (i.e. omasal CP flow equals to CP intake) would be achieved with MUN concentration of 8.3 mg/dl and ruminal NH_4-N concentration of 5.07 mmol/l, and concentrations below this would indicate deficiency of rumen degradable protein. Moreover, the unsupplemented diet in Experiment 2 (I) resulted in highest arterial concentration of Nτ-methylhistidine indicating increased mobilisation of tissue protein reserves. Thus, the long-term sustainability of very low dietary N concentration for dairy cows with high milk production potential may be considered questionable on animal health and welfare perspective.

Interestingly, the average human-edible protein efficiency was decreased with protein supplementation in Experiment 2 (I) (Table 7). However, this change was mostly attributed to the human-edible proportion of diets rather than the effect of protein supplementation itself. Indeed, when comparing only unsupplemented diet to rapeseed meal supplemented diet, rapeseed resulted in numerically lower human-edible protein proportion in the diet (0.232 vs. 0.191 on unsupplemented and rapeseed supplemented diet, respectively) and higher human-edible protein efficiency (Table 7). Thus, it seems that the CP concentration of the diet has only little relevance in terms of human-edible protein efficiency. Indeed, the utilisation of protein sources based on e.g. by-products of food industry have potential to contribute to increasing food

Table 7. Effect of dietary treatments on human-edible protein efficiency (milk protein:human-edible protein intake) in Experiments 1 to 4.

Treatments[1]	Human-edible protein efficiency[2]	SEM	Statistical significance[3]
Exp. 1 (I)			
RSS	0.972	0.1012	LIN: P=0.97
RSS-ALG	1.03		QUAD: P=0.66
ALG	0.978		
Exp. 2 (I)			
NEG	1.48	0.0458	PROTEIN: P<0.001
RSS	1.57		LIN: P<0.001
RSS-ALG	1.09		QUAD: P<0.01
ALG	0.916		
Exp. 3 (II)			
SOY	0.769	0.1499	SOY vs. algae: P=0.054
SPI	1.12		SPI vs. CHL: P=0.25
CHL	0.846		CHL vs. CHL-NAN: P=0.12
CHL-NAN	1.09		
Exp. 4 (III)			
RSM	1.43	0.0334	BP: P<0.001
RSM-SPI	1.03		SPI: P<0.001
FB	0.837		BP × SPI: P<0.001
FB-SPI	0.841		

SEM standard error of the mean. For treatments SOY and CHL-NAN in Experiment 3 SEM must be multiplied by 0.8306 due to missing observations on other treatments.
[1] Exp. 1 and 2: NEG = no protein feed; RSS = rapeseed supplement; ALG = mixture of *Spirulina platensis* and *Chlorella vulgaris* microalgae (Exp. 1) or *S. platensis* (Exp.2); RSS-ALG = mixture of RSS and ALG. Exp. 3: SOY = soya bean meal; SPI = *S. platensis* microalgae; CHL = *C. vulgaris* microalgae; CHL-NAN = mixture of *C. vulgaris* and *Nannochloropsis gaditana* microalgae. Exp. 4: RSM = rapeseed meal; RSM-SPI = rapeseed meal and *S. platensis* microalgae; FB = faba beans; FB-SPI = faba beans and *S. platensis* microalgae.
[2] Human-edible proportions of feeds are calculated based on Wilkinson (2011). For microalgae, human-edible proportions of 0.8 were assumed in line with cereals and pulses.
[3] Exp. 1: LIN and QUAD = linear and quadratic responses to substitution of rapeseed with mixture of *S. platensis* and *C. vulgaris*. Exp. 2: PROTEIN = NEG vs. RSS + RSS-ALG + ALG ; LIN and QUAD = linear and quadratic responses to substitution of rapeseed with *S. platensis* microalgae. Exp. 3: SOY vs. Algae = SOY vs. SPI + CHL + CHL-NAN; SPI vs. CHL = SPI vs. CHL + CHL-NAN; CHL vs. CHL-NAN. Exp. 4: BP = RSM + RSM-SPI vs. FB + FB-SPI; SPI = RSM + FB vs. RSM-SPI + FB-SPI; BP × SPI = interaction between BP and SPI.

security by partially substituting cereals in dairy cow rations, which was also the case in rapeseed supplemented diets.

Microalgae vs. conventional protein feeds

Ruminal concentration of NH_4-N was increased or tended to increase in Experiments 2 (I) and 4 (III), when rapeseed meal was substituted with *S. platensis*, whereas it was decreased by the partial substitution of faba beans with *S. platensis* (Exp. 4; III). This together with similar N intake suggest higher ruminal protein degradability of *S. platensis* than that of rapeseed meal, but lower than that of faba beans. This might also be related to

differences in ruminal microbial synthesis, which tended (P=0.13 for interaction) to increase by *S. platensis* inclusion on faba bean supplemented diets but was not affected on rapeseed. The suggested higher ruminal protein degradability of *S. platensis* than that of rapeseed meal is supported by *in vitro* observations of Costa et al. (2016). However, Wild et al. (2019) reported relatively low *in vitro* degradability values after 8h incubation of four untreated microalgae genera including *Arhtrospira*[1], *Chlorella*, *Nannochloropsis* and *Phaeodactylum* leading to high RUP values (58 to 76% of CP). According to these authors, the intestinal digestibility of RUP of these microalgae was very low, being only 27 to 43% of RUP. Further experiments are needed to confirm these estimates of microalgal ruminal protein degradability because at least on some species of microalgae it seems to be affected by cultivating and harvesting conditions (Lodge-Ivey et al., 2014).

Experiment 1, and Experiments 2 to 4 differed in N intake between diets containing microalgae and conventional protein feeds. In Experiment 1, the substitution of rapeseed meal with microalgae resulted in linear increase in N intake, which was 26 g/d higher on microalgae than on rapeseed supplemented diet. This represents less than 5% of N intake on microalgae diet. The N intake was only 6 and 3 g/d higher on *S. platensis* than rapeseed supplemented diets in Experiments 2 and 4, respectively. The increase in N intake with microalgae inclusion in the diet in Experiment 1 was almost exclusively caused by higher N intake from cereal-based concentrate. The protein from cereals and cereal-based concentrates has higher ruminal protein degradability and lower proportion of metabolisable protein than rapeseed meal (Luke, 2018). Thus, the contribution of the extra protein to metabolisable protein available to milk protein synthesis was likely negligible.

When comparing the effects of rapeseed meal, faba beans and *S. platensis* on NUE, the responses were parallel to that of ruminal NH_4-N with decreasing NUE when *S. platensis* substituted for rapeseed meal (Exp. 2 and 4; I, III) and increasing NUE when *S. platensis* substituted for faba beans (Exp. 4; III). These responses were the consequence of decreased (rapeseed meal supplemented diets) or increased (faba bean supplemented diets) secretion of N into milk.

The excretion of N in urine and faeces did not differ in Experiment 2 (I) between rapeseed meal and *S. platensis* supplemented diets (Figure 8). In contrast, in Experiment 4 (III), the partial substitution of rapeseed meal and faba beans with *S. platensis* increased both urinary and total N excretion on average 13.5 and 15.5 g/d, respectively. This difference was still relatively low considering the average difference of nearly 80 g/d in total N excretion between unsupplemented and protein supplemented diets in Experiment 2

[1] In this thesis, *Spirulina platensis* and *Arthrospira platensis* are considered as synonyms to each other as indicated by Guiry and Guiry (2018). However, the names of other subspecies of these genera might not be used interchangeably. The naming and phylogenetic history of these two cyanobacterial genera has been recently described in Sili et al. (2012), and Furmaniak et al. (2017).

(II). However, in general, simultaneously increased total and urinary N excretion can be considered as the worst outcome, since urinary N is the most susceptible form of N for environmental leaching (Bussink and Oenema, 1998). Interestingly, the opposite was observed in Experiment 3 on microalgae diets in comparison to soya bean meal with decreased urinary N excretion and unaffected total N excretion. In the case of comparing *S. platensis* to rapeseed meal, the increase in urinary N excretion on *S. platensis* can be attributed to suggested higher ruminal protein degradability of *S. platensis* than that of rapeseed meal shifting N metabolism to unproductive purposes. However, the reason for difference on faba bean supplemented diets remain unclear, because it cannot be explained by the ruminal protein degradability, at least if the RDP concentration of *S. platensis* is truly lower than that of faba beans, as is being suggested based on the results of this thesis.

The results of conversion of human-edible protein into milk protein are in favour of rapeseed meal having highest human-edible protein efficiencies especially in Experiments 2 (I) and 4 (III) (Table 7). There are two reasons for this response. First, the milk protein yield was on average numerically slightly higher for rapeseed meal than for other diets. Second, the proportion of human-edible protein in rapeseed meal was assumed to be 20% in these experiments, in contrast to 80% in soya bean meal, faba beans and microalgae according to Wilkinson (2011). Also Karlsson et al. (2018) observed that human-edible protein efficiency was higher for grass silage-based diets constituting of rapeseed meal and sugar beet pulp than for diets constituting of cereals and soya bean meal. In their experiment, cereal-soya bean meal diet resulted in human-edible efficiency of 0.73, which compares reasonably well with the efficiencies obtained for soya bean meal (Exp. 3), faba beans (Exp. 4) and mixture of faba beans and *S.platensis* (Exp. 4) (Table 7). Similarly to many other parameters, the results of Experiment 1 (I) regarding human-edible protein efficiency seem to deviate from other rapeseed experiments in this thesis. This difference seems to centralise on poorer response on rapeseed meal in Experiment 1 than other experiments, since the human-edible protein efficiency of microalgae diets was very stable between and within experiments averaging around 1.

In general, the human-edible efficiencies obtained in Exp. 1 to 4 were relatively low and contradict the review of Dijkstra et al (2013a) summarizing the results of several studies on human-edible protein efficiency of milk production, which were in all cases well over 1 (1.41 to 14.30), i.e. milk production being always the net contributor of human-edible food supply. The extremely high human-edible protein return of 14.30 was achieved with dairy cow diets having 85:15 forage-to-concentrate ratio (CAST, 1999). The results of our experiments and Karlsson et al. (2018) clearly indicate that on diets based on grass silage, cereals and grain legumes such as faba beans or soya bean meal human-edible efficiency can be below 1. Thus, on these diets milk production is the net consumer of human-edible food supply.

When evaluating the sustainability of protein feeds within the scope of this thesis (N utilisation in milk production), it can be concluded that microalgae stands out as slightly inferior to rapeseed meal in terms of NUE, N excretion to environment, and human-edible feed conversion efficiency. However, microalgae performed much better in comparison to grain legumes. Improving the palatability of microalgae diets could also contribute to improved N utilisation, especially when forage and concentrates are fed separately to animals. Gonda et al. (1996) reported decreasing NUE and lower secretion of N in milk when larger part of the dietary N was of forage origin.

Nevertheless, no thorough conclusions can be made from the total sustainability impacts of different protein sources in dairy cow nutrition based on solely the evaluation of N utilisation. The evaluation of single environmental criteria in isolation from others may lead to misinterpretations, because trade-offs can be related to goals to decrease certain environmental loads between or within production phases. For example, the use of maize silage in cattle diets can reduce enteric methane production in comparison to grass silage, but increase greenhouse gas emissions from fields due to increased tillage of soil (Vellinga and Hoving, 2011). Thus, the effects of this feeding strategy can remain negligible in the whole system level (Little et al., 2017). In addition, reducing environmental N emissions of dairy cattle can sometimes result in increases in ruminal methane production (Dijkstra et al., 2011; van Lingen et al., 2018). Therefore, systematic evaluation of dietary interventions on environmental loads with multiple different criteria and metrics covering the effects on whole system is warranted. Without it is very difficult to conclude what are the total environmental benefits and costs of production and utilisation of different protein feeds in dairy cow nutrition.

4.5.2 AMINO ACID METABOLISM

In dairy cow nutrition, different approaches are being used when evaluating adequacy of dietary AA supply and AA limiting milk production. Different approaches can be used to estimate the AA requirements for lactation (Patton et al., 2014). First option is to determine the amount of AA secreted in milk protein and then make assumptions about digestibility and efficiency of utilisation of AA. Second option is to measure the mammary uptake of AA compared to the amount secreted in milk protein. Third option is to assume that there exists an “ideal protein” which has generally AA composition like in casein or microbial protein. In addition, milk and protein yield responses to increased supply of infused AA have been used as an indicator of limiting AA (e.g. Vanhatalo et al., 1999). It has been suggested that plasma concentration of individual EAA would remain steady until the requirements of that EAA have been fulfilled, and increase rapidly after that (Broderick et al., 1974). Thus, the changes in plasma concentrations of individual EAA could be used to determine the EAA limiting milk production. However, this is not supported by Patton et al. (2015), who noticed in their meta-analysis hardly any break

points in plasma concentrations of individual EAA across widely varying duodenal flows of EAA. Instead, the plasma concentrations of EAA increased linearly with increasing duodenal supply. Thus, Patton et al. (2015) concluded that plasma EAA concentrations are not related to their requirements for lactation.

The current experiments were not designed to evaluate the AA limiting milk production on microalgae diets. However, the results can be used to narrow down potential AA that may limit milk production on these diets. Different approaches were utilised in this purpose. Milk protein yield was used as a general indicator to evaluate the quality of protein feeding. In addition, the changes in mammary plasma flow, mammary extraction and uptake of AA in general, and arterial concentrations of AA metabolites were used to evaluate the sufficiency of dietary supply. The decreased methionine supply typically decreases the plasma concentrations of methionine and its metabolites cysteine and taurine, and increases that of serine (e.g. Titgemeyer and Merchen, 1990). The decreased histidine supply, on the other hand, can induce utilisation of endogenous histidine reserves, namely carnosine (β-alanyl-L-histidine) and, to smaller extent, anserine (β-alanyl-Nπ-methylhistidine) from skeletal muscle of cattle (Boldyrev et al., 2012), and blood haemoglobin (Vickery, 1944). The catabolites of carnosine and anserine are β-alanine and histidine, and β-alanine and Nπ-methylhistidine, respectively.

Protein supplementation

Protein supplementation increased the intake and arterial concentration of several EAA in Experiment 2 (I), which is in agreement with Choi et al. (2002) and Korhonen et al. (2002) also having grass silage-based diets. These authors reported increased omasal concentration (Choi et al., 2002) and flow of non-NH_4-N (Choi et al., 2002; Korhonen et al., 2002) with protein supplementation. This non-NH_4-N constituted mainly of free AA (around 40%) but to some extent also peptides and protein (Choi et al., 2002). Also Korhonen et al. (2002) reported increasing omasal flows of many individual AA with protein supplementation. Patton et al. (2015) concluded in their meta-analysis that plasma AA concentrations increased linearly with predicted duodenal flow over widely varying protein intake levels. Thus, it can be assumed that protein supplementation in Experiment 2 (I) increased duodenal supply of EAA, which subsequently increased their arterial concentrations. This is logical given the increased total tract apparent digestibility of DM, OM, NDF and CP by protein supplementation.

Microalgae vs. conventional protein feeds

The substitution of rapeseed meal, soya bean meal and faba beans with microalgae resulted in decreased intake of histidine but increased intake of methionine in Exp. 1 to 4 (I-III). This was expected, because microalgae are typically rich in methionine but poor in histidine when compared to conventional protein feeds (Figure 4). Histidine and methionine are the most

interesting AA considering microalgae feeding to dairy cows, because shortages of these AA are commonly reported in dairy cow diets. Histidine is typically the first AA limiting milk production on cereal and grass silage-based diets (Kim et al., 1999; Vanhatalo et al., 1999), and corn silage-based diets deficient in metabolisable protein (Lee et al., 2012). On the other hand, leguminous feeds such as soya bean meal and faba beans are generally low in methionine (Figure 4), thus this AA is considered the most limiting AA on soya bean meal supplemented diets (Casper and Schingoethe, 1988; Pisulewski et al., 1996), and potentially the most limiting on faba bean supplemented diets (Puhakka et al., 2016). Rapeseed being high in both histidine and methionine (Maxin et al., 2013a) is therefore considered well suited to supplement dairy cow diets (Huhtanen et al., 2011; Martineau et al., 2013).

The supply of methionine and histidine from microalgal diets had varying responses to arterial concentrations of these AA and their metabolites. The substitution of rapeseed meal with *S. platensis* (Exp. 2; I) or mixture of *S. platensis* and *C. vulgaris* (Exp. 1; I) decreased arterial concentrations of histidine and carnosine, but did not affect other histidine metabolites in arterial blood. As also mammary uptake of histidine and milk protein yield tended to decrease in Exp. 2 (I), it was concluded that histidine supply may become suboptimal on microalgae diets based on grass silage and cereals. In Experiment 1, the microalgal shortage of histidine was might have been partially compensated by linearly increased CP and AA intake when rapeseed meal was substituted with microalgae if the extra CP and AA were converted to metabolisable protein available for intestinal absorption. The complete substitution of rapeseed meal with microalgae resulted in 5.2% decrease in histidine intake in Experiment 1, whereas in Experiment 2 it was 7.2%. Correspondingly, the partial substitution of rapeseed meal with microalgae decreased histidine intake by 1.1% in Exp. 1, 5.0% in Exp. 2 and 3.1% in Exp.4. Together with unaffected composition and quantity of DMI this might explain why milk protein yield was not affected by microalgae inclusion in the diet in Experiment 1 similarly as in other experiments with rapeseed meal as a control. Increased AA intake has also potential to increase DMI by increasing the clearance rate of metabolic fuels from blood leading to decrease in satiety (Allen, 2000). Despite of decreased histidine intake, the complete substitution of soya bean meal with different microalgae, or the partial substitution of rapeseed meal or faba beans with *S. platensis* did not have any effects on arterial concentration of histidine or its metabolites. It is possible that lack of dietary histidine supply will not become apparent in short-term experiments due to the utilisation of endogenous histidine reserves. Lapierre et al. (2012) estimated the carnosine reserves of dairy cow to be approximately 420 g; however, it is unknown how long these reserves can provide sufficient histidine supply to lactating dairy cow. Thus, the results regarding histidine sufficiency and metabolism on microalgae diets remain inconclusive and further research is warranted on the long-term effects of microalgae feeding on milk production and AA metabolism.

The lack of arterial methionine response on increased methionine supply by microalgae inclusion in the diets is worth noting. For example in Experiment 4 (III), rapeseed meal supplemented diets resulted in on average 9.35 g/d higher methionine intake than faba bean supplemented diets. Methionine intake was further increased on both rapeseed meal and faba bean supplemented diets (1.5 and 4.9 g/d, respectively) by the inclusion of *S. platensis* in the diets. The higher methionine supply on rapeseed meal than on faba beans induced the classical responses of methionine and methionine metabolites in arterial plasma with increasing methionine, cysteine and taurine, and decreasing serine concentration. Indeed, rapeseed meal have been suggested to supply greater amount of metabolisable protein (Maxin et al., 2013b) and increase absorption of AA in comparison to soya bean meal (Shingfield et al., 2003; Maxin et al., 2013b). However, even though methionine intake was increased also when *S. platensis* partially substituted for rapeseed meal and faba beans, dietary AA supply did not convert to absorbed AA similarly as in rapeseed meal supplemented diets, as not any effects were seen on arterial concentrations of methionine or methionine metabolites. The same was true for microalgae diets also in Experiments 1 to 3 (I-II).

One possible reason for this might be the suggested higher ruminal protein degradability of microalgae than that of rapeseed meal, or possibly low intestinal digestibility of microalgae protein as suggested by Wild et al. (2019). The first would lead to lower amounts of undegraded methionine flow to intestines, and the latter would compromise the absorption of methionine despite of initially larger dietary supply. This is also supported by the results of arterial concentrations of BCAA, which remained unaffected in Experiments 2 and 4 despite of their increased intake when rapeseed meal was substituted with *S. platensis*. In contrast to other AA, BCAA are not extensively degraded in the liver after absorption from small intestine (Harper et al., 1984), therefore, changes in their plasma concentration can be used as an indicator of AA absorption (Bergen et al., 1973). However, further experiments are needed to determine the ruminal degradability and intestinal digestibility of microalgal protein in relation to conventional protein feeds, and the effects of microalgal cultivation and harvesting practices on these characteristics.

5 CONCLUSIONS

1. The CP concentration of *S. platensis* and *C. vulgaris* was very high in comparison to conventional protein feeds, which is in agreement with literature. The CP concentration of *N. gaditana* was comparable to rapeseed and soya bean feeds in current experiments. These non-defatted microalgae were comparably low in crude fat considering the literature values reported for other species of microalgae. The NDF concentration of microalgae was generally markedly lower than in conventional protein feeds, however, the results indicated high sensitivity to crucible pore size used in the analysis. Therefore, further development of standard analytical methods is warranted for the fibre analysis of unicellular microalgae with very small particle size.

2. The intake of microalgae containing diets was lower than that of rapeseed meal, soya bean meal and faba beans. This was demonstrated in three out of four experiments. In separate feeding of concentrates and forages, cows compensated decreased intake of microalgae containing concentrates by increasing silage intake. This led to unchanged total DMI. When cows no longer were able to avoid microalgae in TMR feeding, total DMI was decreased when rapeseed meal was partially substituted with *S. platensis*. The differences in intake between experiments were not related to the amount of microalgae in the diet, dietary crude fat concentration or stage of lactation. Instead, microalgae intake was constrained mostly by the poorer palatability relative to conventional feeds, which could be related to sensory characteristics or mineral, especially Na, concentration of microalgae.

3. The effect of substitution of conventional protein feeds with microalgae on milk production depended on the source of protein in the basal diet. The slight inferiority of microalgae to rapeseed meal in dairy cow diets was demonstrated in one out of three experiments by lower milk yield on microalgae than rapeseed diets. Moreover, in two experiments, microalgae tended to result in lower milk protein yield than rapeseed meal. However, microalgae were considered suitable substitute for grain legumes as indicated by milk production responses. Microalgae resulted in milk, ECM and protein yields similar to soya bean meal and increased the milk yield on faba bean supplemented diets. The milk fat promoting effect of *S. platensis* observed in two out of four experiments might be related to more acetate intensive ruminal fermentation, increased body lipid mobilisation or higher methionine intake.

4. The results of this thesis are in line with the general agreement that protein supplementation decreases NUE and increases total N excretion to environment. Microalgae were slightly inferior to rapeseed meal as judged by lower NUE, higher total and urinary N excretion to environment, and lower human-edible protein efficiency. Different N metrics reflected the inconsistencies of substituting grain legumes with microalgae related to N utilisation. Compared to soya bean meal, microalgae resulted in similar NUE and total N excretion, lower urinary N excretion, and a tendency for higher human-edible protein efficiency. On the other hand, the partial substitution of faba beans with *S. platensis* increased NUE, total and urinary N excretion, but did not affect human-edible protein efficiency.

5. The substitution of all conventional protein feeds with microalgae decreased the intake of histidine and increased the intake of methionine. However, in three out of four experiments, these changes did not convert to expected changes in arterial concentrations of these AA or their metabolites. Further research is needed on ruminal protein degradability and intestinal digestibility of microalgae. These characteristics can greatly influence the intestinal availability and absorption of AA.

6. The results of this thesis demonstrated that there are no biological or physiological constraints to protein feed use of different microalgae species for lactating dairy cows. However, the economically reasonable production cost of microalgae is the prerequisite for the large-scale utilisation of microalgae in the livestock nutrition. Based on results of this thesis, microalgae are suitable substitute for grain legumes in dairy cow nutrition, but seemed to be slightly inferior in comparison to rapeseed meal. Nevertheless, no thorough conclusions can be made from the total sustainability impacts of different protein sources in dairy cow nutrition based on solely the evaluation of N utilisation in milk production.

7. The greatest challenge limiting the feed use of microalgae for lactating dairy cows is the poorer palatability relative to conventional feeds, which was demonstrated in both separate feeding of concentrates and forage, and TMR feeding. However, improving the palatability of microalgae e.g. by feed processing or deodorisation of microalgae would likely markedly improve also the milk production responses of microalgae diets, given that DMI is the main factor affecting milk yield.

6 FUTURE RESEARCH

The general lack of knowledge related to feed value, palatability and high production costs of microalgae currently limits the feed use of microalgae in dairy cow diets. As feed intake is linked to both animal welfare, productivity and nutrient use efficiency, improving the palatability of microalgae e.g. by feed processing or different feeding strategies should be considered as the key aim of the future research on microalgae with ruminant animals. The feeding strategy can also greatly contribute to decreasing production costs of microalgae, especially if microalgae is given to animals via drinking water or mixed to TMR as wet paste to avoid the expensive drying phase. In order to use fresh microalgae on animal nutrition, microalgae should be produced in the close proximity of the livestock farm, which could also create jobs and new sources of income to the livestock production regions.

Further research is also warranted on the digestibility, ruminal protein degradability and kinetics of microscopic microalgae that may not perform in the rumen similarly to conventional feeds. Systematic research is needed to evaluate the effects of the cultivation conditions and harvesting methods of microalgae to its nutritive value, digestibility and protein degradability. The better understanding of these responses increases the replicability of research findings and enables the optimisation of microalgae composition for different purposes in livestock nutrition. Research on *in vitro* models could greatly contribute to the digestibility and rumen degradability values of microalgae. However, research on animal models is a necessity to confirm these results as sometimes the responses in animals differ greatly from laboratory models. The question is: can microalgae with very small particle size and density close to that of water flow through rumen in liquid phase and how does this affect the digestibility and ruminal protein degradability of microalgae if its retention time in rumen is significantly lower than that of conventional feeds? Does this shift the absorption of microalgal nutrients to lower parts of digestive tract?

Due to the relative high production costs of microalgae, livestock feeds will unlikely consist of intact microalgae, but different kinds of microalgae residues as by-products or side-streams from other industries. Alternatively, microalgae can be used in small targeted doses as feed additives to deliver certain active ingredients such as antioxidants aiming to improve animal health or the nutritive value of animal products. The integration of livestock feed production with e.g. the purification of wastewaters or flue gases, production of biofuels, and extraction of different bioactive compounds for medical or cosmetic purposes from microalgae is another field of research that should be given more attention. It is important to quantify the possible contaminants from these processes that may impair the feeding value or even intoxicate the resulting microalgae residue. Moreover, at least some lipid extraction methods may increase the ash concentration of residual microalgae

biomass in comparison to intact microalgae (He et al., 2017). This is highly relevant considering the feed use of microalgae, because microalgal mineral and especially Na concentration was suggested to be one potential reason for lower palatability of microalgae than conventional protein feeds.

Originally, microalgae production was thought to be economically feasible only on tropic or sub-tropic areas close to equator (Adenle et al., 2013). However, the fast technological development (e.g. energy efficient led lights) has enabled the microalgae production also in colder areas when cheap renewable energy is available. Large part of the commercial microalgae production currently focuses on few species of relatively well known microalgae (Usher et al., 2014). However, new species adapted to colder climates might be needed for microalgae production in temperate or even boreal regions. Therefore, research is warranted on the characteristics, growth rate and nutritive value of microalgae species and genera growing in cold natural habitats e.g. in Baltic Sea.

The sustainability evaluation of feeds and animal feeding in general is very challenging because of the lack of holistic evaluation tools and the existence of multiple different affecting factors, many of which interact or counteract with each other. Indeed, the sustainability effects of even the conventional protein feeds are not completely understood in the whole milk production chain. As trade-offs can be related to different practices to decrease environmental load of livestock production, research should evolve from focusing only on single environmental pollutant to simultaneous evaluation of multiple different pollutant sources (e.g. greenhouse gases, N and P) within and between different phases in food production chain.

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